Investigation of the Role of Glial Cells in Prion Toxicity and a Potential Function of PrP^C as a Cell Death Transducer

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1. Summary

Prion diseases are fatal neurodegenerative disorders that can affect humans as well as a broad range of mammals (Aguzzi and Calella, 2009). The disease causing agent is the prion, a misfolded form of the cellular prion protein PrP^C (Aguzzi and Calella, 2009). Apart from the fact that PrP^C is an essential neuronal scaffolding protein (Bueler et al., 1993; Weissmann et al., 1994), the molecular mechanism underlying neurodegeneration in prion diseases is poorly understood (Aguzzi and Calella, 2009).

Research on the neurodegenerative mechanisms of prion disease is hampered by the difficulty in propagating prions in cell lines (Rubenstein et al., 1984; Race et al., 1987; Butler et al., 1988; Schatzl et al., 1997; Bosque and Prusiner, 2000; Milhavet et al., 2000). In fact, even if propagation occurs, neurotoxic effects similar to those in prion diseases are not observed (Vilette, 2008).

The aim of my first project was to assess whether the presence of glial cells would make neurons vulnerable to prion mediated toxicity. This approach was based on the observation that non-cell autonomous interactions with glial cells are crucial for neuronal loss in many neurodegenerative diseases (Lobsiger and Cleveland, 2007; Ilieva et al., 2009). In particular, I was interested in the non-cell autonomous effect of astrocytes because the accumulation of PrP^{Sc} appears at early stages of the disease (Diedrich et al., 1991) and significant changes in astrocytes occur during disease progression (Eklund et al., 1967). In addition, prion replication in astrocytes is sufficient to indirectly induce neurodegeneration *in vivo* (Raeber et al., 1997; Jeffrey et al., 2004).

Previous results from our lab suggested that POM1, an antibody that mimics prion mediated toxicity, triggers neurodegeneration in mouse primary cortical neurons overexpressing PrP^C only in presence of astrocyte conditioned media (ACM). However, these data were not reproducible. A robust readout was established with the almarBlue Assay cell viability assay that is based on the reducing environment of a viable cell (Nociari et al., 1998; Rampersad, 2012). It was observed that ACM is not toxic to primary cortical neurons overexpressing PrP^C or alters expression pattern of a set of immediate early genes (IEG) associated with neurodegenerative disease and/or synaptic plasticity.

ACM alone was not sufficient to trigger neuronal loss upon POM1 treatment. Therefore, astrocyte neuron co-cultures were used for further experiments. The alamarBlue assay is not suitable to detect neuron specific cell death (Nociari et al., 1998; Rampersad, 2012); therefore, live cell imaging of fluorescently labeled neurons that allows defining early sign of neurodegeneration based on changes in neuronal morphology and number of surviving cells were performed. In particular, mouse embryonic stem cell (ESC) derived moto-, inter- and

pan neurons were used, which express wild type PrP^C in co-culture with astrocytes. In none of these different neuronal populations POM1 mediated neurodegeneration was observed.

Translational changes upon POM1 treatment were examined in human induced pluripotent stem cell (iPSC) derived pan neuronal cultures in sandwich culture with astrocytes. The expression level of proteins affected in prion disease did not reveal any changes upon POM1 treatment.

Since PrP^C expression in astrocytes was sufficient for prion induced neuronal loss (Raeber et al., 1997; Jeffrey et al., 2004), the potential of astrocytes to become reactive after POM1 treatment was tested. The transcriptional level of the reactive astrocyte markers LCN2 (lipocalin-2) and GFAP (glial fibrillary acidic protein) (Zamanian et al., 2012) did not change after the application of POM1 to astrocytes and astrocyte/microglial co-cultures.

In summary under the conditions studied, astrocytes were not able to render neurons vulnerable to POM1 mediated toxicity *in vitro*. It is possible that astrocytes alone are not sufficient or other structural aspects are needed to induce POM1 mediated cell death.

POM2 an antibody against the flexible tail of PrP^C is able to counteract POM1 and prion mediated toxicity (Sonati et al., 2013; Herrmann et al., 2015). In particular, it has been shown that the PERK (protein kinase RNA-like endoplasmic reticulum kinase) signaling branch of the unfolded protein response (UPR) is activated in POM1 and prion mediated toxicity, whereas POM2 treatment reduces this effect (Herrmann et al., 2015). Activation of the UPR upon ER stress is common to many neurodegenerative diseases (Hetz and Mollereau, 2014). Therefore, the aim of my second project was to examine the role of PrP^C as a general cell death transducer. To assess cell viability, live cell imaging was performed followed by morphometric analysis and measurement of the cell number with significant outgrowth. There are indications that POM2 can delay ER stress induced in mouse primary granule neurons overexpressing PrP^C. POM2 was not able to counteract ER stress in PrP^C depleted neurons suggesting that this effect is PrP^C dependent.

iPSC derived human neurons expressing wild type PrP^C were exposed to POM2 before ER stress induction to examine if POM2 induces translational changes in the UPR. Upon strong induction of ER stress PrP^C levels increased. Similar results have been already shown in a cancer cell line and in primary human neurons (Dery et al., 2013; Misiewicz et al., 2013), which support our hypothesis that PrP^C is crucial for UPR signaling induced via ER stress. However, application of POM2 did not affect PrP^C expression, which might be due to the strong induction of ER stress. ER stress induced by dysregulation of calcium homeostasis indicates that POM2 delays the activation of BiP, ATF4 and CHOP, which are involved in the PERK signaling branching affected in prion disease (Herrmann et al., 2015).

As a next step, the therapeutic potential of POM2 *in vivo* should be elucidated. My preliminary data are consistent with previous results from the lab and suggest that the intravenous application of POM2, which causes hemolysis, is not suitable for antibody delivery. Since it is assumed that hemolysis is mediated via complement activation, Dr. Vijay Chandrasekar designed an adeno associated virus (AAV) overexpressing the scFv POM2 antibody lacking the Fc fragment responsible for complement activation and the fluorescent marker tdTomato. It was shown that the virus can efficiently transduce cerebellar brain slices and secreted POM2. Before testing the protective potential of the virus in complex *in vivo* model systems of neurodegenerative diseases, the virus was used to counteract photoreceptor mediated apoptosis in the mouse retina. This represents a simpler model system with a faster read out. Light induced phototoxicity causes photoreceptor loss in PrP^C wildtype and PrP^C depleted Balb/c retinas. However neither the expression of PrP^C nor the transduction of AAV8 scFv POM2 could attenuate phototoxicity. One explanation could be that transduction was ineffective because cells were not labeled with the fluorescent marker tdTomato after infection. In addition, light induced photoreceptor toxicity activates the caspase-1 pathway (Grimm et al., 2000); whereas recent results suggest that prion mediated toxicity is calpain dependent (Sonati et al., 2013; Herrmann et al., 2015). Based on the different molecular mechanism leading to cell loss, the role of PrP may change.

In summary, POM2 delays ER stress induced neurodegeneration; however, further studies will be necessary to confirm these results.

2. Zusammenfassung

Prionenerkrankungen sind zum Tode führende neurodegenerative Erkrankungen, die sowohl bei Menschen als auch einer Vielzahl von Säugetieren auftreten können (Aguzzi and Calella, 2009). Das krankheitserregende Agens ist das Prion (PrP^{Sc}), eine fehlgefaltete Form des zellulären Prion-Proteins (PrP^{C}) (Aguzzi and Calella, 2009). Abgesehen von der Tatsache, dass PrP^{C} ein essentielles neuronales Gerüstprotein ist (Bueler et al., 1993; Weissmann et al., 1994), ist der krankheitsunterliegende molekulare Mechanismus in Prionenerkrankungen unzureichend verstanden (Aguzzi and Calella, 2009).

Die Erforschung des neurodegenerativen Mechanismus in Prionenerkrankungen wird dadurch erschwert, dass sich Prionen in Zellkulturen nur eingeschränkt propagieren lassen (Rubenstein et al., 1984; Race et al., 1987; Butler et al., 1988; Schatzl et al., 1997; Bosque and Prusiner, 2000; Milhavet et al., 2000). Selbst wenn eine Propagation stattfindet, können keine eindeutigen neurotoxischen Effekte an den Zellen beobachtet werden, die einer Prionerkrankung *in vivo* ähneln (Vilette, 2008).

Das Ziel meines ersten Projektes war die Bewertung, ob in Gegenwart von Gliazellen Neuronen für Prionen vermittelte Toxizität anfällig werden. Diese Herangehensweise basiert auf der Beobachtung, dass nicht zellautonome Interaktionen mit Gliazellen wesentlich zum neuronalen Zellverlust in vielen neurodegenerativen Erkrankungen beitragen (Lobsiger and Cleveland, 2007; Ilieva et al., 2009). Insbesondere war ich interessiert am nicht zellautonomen Effekt von Astrozyten, da die Akkumulation von PrP^{Sc} im frühen Stadium der Krankheit stattfindet (Diedrich et al., 1991) und signifikante Veränderungen der Astrozyten während des Krankheitsverlaufs auftreten (Eklund et al., 1967). Ausserdem ist eine Prionenreplikation in Astrozyten ausreichend, um eine indirekte Neurodegeneration *in vivo* auszulösen (Raeber et al., 1997; Jeffrey et al., 2004).

Vorhergehende Ergebnisse von unserem Labor legen nahe, dass POM1, ein Antikörper der Prionentoxizität nachahmt, nur in Gegenwart von Astrozyten konditioniertem Medium (ACM) zur Neurodegeneration in primären kortikalen Neuronen von Mäusen, welche PrP^{C} überexprimieren, führt. Jedoch waren diese Daten nicht reproduzierbar. Ein robuster Readout wurde etabliert mit dem almarBlue Zellviabilitätsassay, welches auf dem reduzierenden Umfeld einer lebenden Zelle basiert (Nociari et al., 1998; Rampersad, 2012). Es wurde beobachtet, dass ACM nicht toxisch auf primäre kortikale Neuronen wirkt, welche PrP^{C} überexprimieren, oder das Expressionsmuster von einem Set von Immediate Early Genes (IEG), welche mit neurodegenerativen Erkrankungen und/oder synaptischer Plastizität assoziiert sind, verändert.

ACM alleine war nicht ausreichend um einen neuronalen Zellverlust durch POM1 Behandlung auszulösen. Daher wurden Co-Kulturen von Astrozyten und Neuronen für

weitere Experimente verwendet. Der almarBlue Assay ist nicht geeignet um neuronenspezifischen Zelltod zu detektieren (Nociari et al., 1998; Rampersad, 2012). Daher wurde Live Cell Imaging von Fluoreszenz markierten Neuronen durchgeführt, welches die Bestimmung von frühen neurodegenerativen Veränderung in Form von neuronaler Morphologie und Anzahl der überlebenden Zellen ermöglicht. Dafür wurden eine Derivation von embryonalen Stammzellen (ESCs) der Maus in Moto-, Inter- und pan neuronale Kulturen durchgeführt, welche Wildtyp PrP^C exprimieren. Diese wurden in Ko-Kultur mit Astrozyten gesetzt. In keiner dieser unterschiedlichen Populationen konnte eine POM1 vermittelte Neurodegeneration beobachtet werden.

Humanen induzierte pluripotenten Stammzellen (iPSC) derivierte pan neuronalen Kulturen in Sandwich Kultur mit Astrozyten wurden verwendet, um translationale Veränderungen ausgelöst durch POM1 Behandlung zu untersuchen. Das Expressionslevel von Proteinen, die in Prionenerkrankungen betroffen sind, war nicht verändert.

Da PrP^C Expression in Astrozyten ausreichend ist, um neuronalen Verlust zu verursachen (Raeber et al., 1997; Jeffrey et al., 2004), wurde das Potential von Astrozyten nach POM1 Behandlung reaktiv zu werden untersucht. Die transkriptionellen Level von den Astrozytenmarkern LCN2 (lipocalin-2) und GFAP (glial fibrillary acidic protein) (Zamanian et al., 2012) waren nicht verändert nach der Applikation von POM1 zu Astrozyten und Astrozyten/Mikrogila Ko-Kulturen.

Zusammenfassend fanden sich keine Hinweise, dass unter den betrachteten Konditionen Astrozyten einen additiven POM1 vermittelten neurotoxischen Effekt auslösen. Es ist möglich, dass Astrozyten allein nicht ausreichen oder strukturelle Aspekte notwendig sind um POM1 vermittelten Zelltod *in vitro* zu induzieren.

POM2, ein Antikörper gegen den flexiblen Schwanz von PrP^C, ist fähig POM1 und Prion vermittelter Toxizität entgegenzuwirken (Sonati et al., 2013; Herrmann et al., 2015). Im Speziellen wurde gezeigt, dass der PERK (protein kinase RNA-like endoplasmic reticulum kinase) Signalzweig des Unfolded Protein Response (UPR) in POM1 und Prionen vermittelter Toxizität aktiviert ist, wohingegen POM2 diesem Effekt entgegenwirkt (Herrmann et al., 2015). Aktivierung des UPR durch ER Stress tritt in vielen neurodegenerativen Erkrankungen auf (Hetz and Mollereau, 2014). Daher war das Ziel meines zweiten Projektes die Rolle von PrP^C als generellen Zelltodvermittler zu untersuchen. Um die Zellviabilität zu beurteilen, wurde Live Cell Imaging gefolgt von morphometrischer Analyse und Messung der Zellzahl mit signifikantem Auswuchs durchgeführt. Es gibt Hinweise, dass POM2 ER Stress in primären PrP^C überexprimierenden Granularzellen der Maus, verzögern kann. POM2 war nicht fähig, ER Stress in PrP^C knock out Neuronen entgegenzuwirken. Dies deutet darauf hin, dass dieser Effekt PrP^C abhängig ist.

iPSC derivatisierte humane Neuronen, welche Wildtyp PrP^C exprimieren, wurden vor der Induktion von ER Stress mit POM2 behandelt, um zu untersuchen, ob POM2 translationale Veränderungen im UPR auslöst. Die PrP^C Expression war nach starker Induktion von ER Stress erhöht. Ähnliche Ergebnisse wurden bereits in einer Krebszelllinie und in primären human Neuronen gezeigt (Dery et al., 2013; Misiewicz et al., 2013). Dies unterstützt unsere Hypothese, dass PrP^C essentiell für die ER Stress induzierte UPR Signalisierung ist. Allerdings konnte die Applikation von POM2 die PrP^C Expression nicht beeinflussen, was möglicherweise an der starken Induktion von ER Stress liegt. ER Stress, induziert durch Fehlregulierung der Calcium Homöostase, deutet an, dass POM2 die Aktivierung von BiP, ATF4 und CHOP verzögert, welche in dem PERK Signalzweig involviert sind, welcher in Prionenerkrankungen betroffen ist (Herrmann et al., 2015).

Als nächster Schritt sollte das therapeutische Potential von POM2 *in vivo* bewertet werden. Meine vorläufigen Daten stimmten mit vorherigen Ergebnissen aus dem Labor überein und deuten an das intravenöse Applikation von POM2 Hämolyse verursacht und deshalb nicht für die Antikörperzufuhr *in vivo* geeignet ist. Da angenommen wird, dass Hämolyse durch Komplementaktivierung verursacht wird, hat Dr. Vijay Chandrasekar einen Adeno Assoziierten Virus (AAV) generiert, der scFv POM2, ein Antikörper ohne Fc Fragment, welches für Komplementaktivierung verantwortlich ist und den fluoreszierenden Marker tdTomato überexprimiert. Es wurde gezeigt, dass der Virus effizient zerebrale Gehirnschnitte transduzieren kann und POM2 sekretiert. Bevor das protektive Potential des Virus in komplexen *in vivo* Modelsytemen von neurodegenerativen Erkrankungen getestet wird, wurde der Virus verwendet, um Photorezeptor vermittelte Apoptose in der Mausretina entgegenzuwirken. Dies stellt ein einfacheres Modelsystem mit einem schnelleren Ausleseverfahren dar. Licht induzierte Phototoxizität führte zu Photorezeptoren-Verlust in der Retina von PrP^C Wildtyp und PrP^C knockout Balb/c Mäusen. Jedoch konnte weder die Expression von PrP^C noch die Infektion von AAV8 scFv POM2 die ausgelöste Toxizität abschwächen. Eine Erklärung hierfür könnte eine ineffektive Transduktion sein, da der fluoreszierende Marker tdTomato nach der Infektion nicht nachgewiesen wurde. Ausserdem ist bei Licht induzierter Photorezeptor Toxizität der Caspase-1 Signalweg aktiviert (Grimm et al., 2000), wohingegen kürzlich publizierte Ergebnisse darauf hindeuten, dass Prionen vermittelte Toxizität Calpain abhängig ist (Sonati et al., 2013; Herrmann et al., 2015). Basierend auf unterschiedlichen molekularen Mechanismen, die zum Zelltod führen, könnte die Funktion von PrP verändert sein.

Zusammenfassend verzögert POM2 ER Stress induzierte Neurodegeneration. Jedoch sind weitere Studien nötig, um das Ergebnis zu bestätigen.

3. Abbreviation

Aβ	Amyloid beta
7ADD	7-Aminoactinomycin D
ACM	astrocyte conditioned media
ALS	Amyotrophe Lateral Sclerosis
AM	astrocyte media
ATF4	activating transcription factor 4
ATF6	activating transcription factor 6
B2m	Beta-2-Microglobulin
BDNF	brain-derived neurotrophic factor
BSE	bovine spongiform encephalopathy
CC1	small charge cluster one domain
CC2	charge cluster two domain
CDP	chronic demyelinating polyneuropathy
CHOP	C/EBP homologous protein
CMV	cytomegalovirus
CNTF	ciliary neurotrophic factor
CREB	cAMP response element binding protein
CWD	Chronic wasting disease
DAPI	4′,6-diamidino-2-phenylindole
Dpl	Doppel
EB	embryoid body
Egr1	Early growth response protein 1
eIF2α	Eukaryotic translation initiation factor 2α
ER	Endoplasmic Reticulum
ERK1/2	extracellular signal-regulated kinases 1 and 2
FACS	fluorescence-activated cell sorting
FFI	Fatal Familial insomnia
FSC	forward scatter
FTgpi	membrane-anchored version of the flexible tail of PrP^C
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GCL	ganglion cell layer
GDNF	glial cell-derived neurotrophic factor

GFAP	glial fibrillary acidic protein
GPI	glycosyl phosphatidyl inositol
GSS	Gerstmann-Sträussler-Scheinker
Gusb	glucuronidase beta
HB9	homeodomain transcription factor 9
HC	hydrophobic core
Hmbs	hydroxymethylbilane synthase
HRP	horse radish peroxidase
IEG	Immediate Early Genes
INL	inner nuclear layer
IPL	inner plexiform layer
LCN2	lipocalin-2
LIF	leukemia inhibitory factor
MAPK	Mitogen-associated protein kinases
mESC	mouse embryonic stem cells
mGluR5	metabotropic glutamate receptor 5
NAD+	nicotinamide adenine dinucleotide (oxidized form)
NADH	nicotinamide adenine dinucleotide (reduced form)
NT-3	neurotrophin-3
ONL	outer nuclear layer
OPL	outer plexiform layer
OR	octapetide region
P/S	penicillin/streptomycin1
PBS	phosphate buffered saline
PERK	Protein Kinase RNA-like Endoplasmic Reticulum Kinase
PMD	protein misfolding diseases
POSCA	prion organotypic slice culture assay
Ppib	peptidylprolyl isomerase B
PrP	prion protein
PrP^{C}	cellular prion protein
PrP^{Sc}	prion scrapie
qRT-PCR	quantitate real time polymerase chain reaction
RA	retinoic acid
RPE	retina pigmented epithelium

rPrP	recombinant prion protein
SAG	Smoothed agonist
sCJD	sporadic Creutzfeld-Jakob disease
SD	Standard deviation
SNP	single nucleotide polymorphism
SP	signal peptide
SSC	side scatter
ST	Staurosporine
Tbp	TATA-binding protein
TBS	tris buffered saline
TH	Thapsigargin
TM	Tunicamycin
TMB	3,3',5,5'-Tetramethylbenzidin
TSE	transmissible spongiform encephalopathy
UPR	Unfolded Protein Response
VBA	Visual Basic Application
vCJD	variant Creutzfeld-Jakob disease

4. General introduction to prion disease

4.1. Prion disease

Prion diseases, also known as transmissible spongiform encephalopathies (TSEs), are caused by infectious self-replicating protein units (Kovacs and Budka, 2008). Prion diseases are fatal neurodegenerative disorders that affect humans and a broad range of mammals (Aguzzi and Calella, 2009). Prion diseases can be divided into genetic, acquired and sporadic forms, according to their etiology (Murdoch and Murdoch, 2015). The most striking feature of prion pathology is the spongiform degeneration of the brain (Budka, 2003; Kovacs and Budka, 2008). Other characteristics are neuroinflammation, synaptic impairment and the formation of plaques (Soto and Satani, 2011) (see Figure 1). Disease progression and symptoms depend on the trigger of the prion disease and the genotype involved (Aguzzi and Calella, 2009). Common clinical characteristics are dementia, cerebellar ataxia and motor dysfunction (Aguzzi and Calella, 2009).

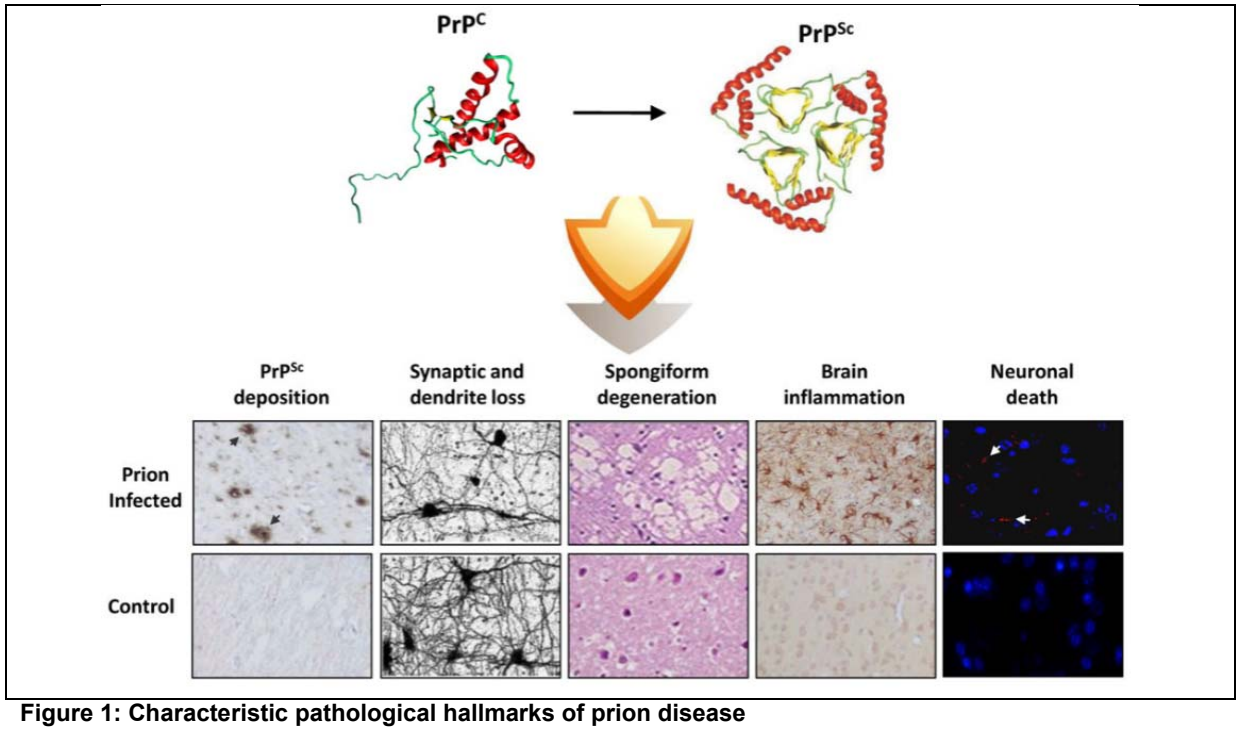


Figure 1: Characteristic pathological hallmarks of prion disease

In prion disease PrP^{C} is converted into its misfolded conformer PrP^{Sc} (Soto and Satani, 2011). The pathological features can be can be visualized by different staining techniques (Soto and Satani, 2011). PrP^{Sc} depositions are identified by immunohistochemistry with PrP specific antibodies (Soto and Satani, 2011). Silver staining is used to characterize the synaptic and dendrite loss (Soto and Satani, 2011). Spongiform degeneration is assessed by hematoxylin and eosin staining (Soto and Satani, 2011). Inflammation is characterized by immunohistochemistry with an anti-GFAP (glial fibrillary acidic protein) antibody that labels reactive astrocytes (Soto and Satani, 2011). Neuronal loss is visualized by caspase-3 antibody (red) and DAPI (4′,6-diamidino-2-phenylindole, blue) staining of nucleus (Soto and Satani, 2011).
(Image: (Soto and Satani, 2011))

The infectious agent, PrP^{Sc}, is a misfolded form of the cellular prion protein PrP^{C} (Prusiner, 1998). Following conversion, PrP^{Sc} molecules can aggregate and recruit PrP^{C}, supporting their own amplification (Aguzzi and Calella, 2009).

It was previously thought that prion diseases are the only transmissible neurodegenerative diseases (Glatzel et al., 2005). Howeve,r there is now growing evidence that other protein misfolding diseases (PMDs) like Alzheimer's disease, Parkinson and Huntington's disease are also transmissible (Aguzzi and Rajendran, 2009; Ashe and Aguzzi, 2013). Importantly, while many misfolded proteins involved in PMDs have been shown to be transmissible, they are not infectious which discriminates them from bona fide prions (Aguzzi and Lakkaraju, 2016). Therefore Prof. Aguzzi has introduced the term "prionoids" to describe these propagons (Aguzzi and Rajendran, 2009; Ashe and Aguzzi, 2013).

As for many other neurodegenerative disorders, the chain of events that leads to neuronal loss is not resolved (Aguzzi and Falsig, 2012; Chiesa, 2015). However, it is known that PrP^{C} expression is essential for prion-mediated neuronal loss (Bueler et al., 1993; Sailer et al., 1994). Moreover neuron-specific PrP^{C} depletion in mice prohibits PrP^{Sc} induced neuro-toxicity (Race et al., 1995; Mallucci et al., 2003).

4.1.1. Prion disease in animals

In the 18th century prion disease was first recognized in sheep, and was termed scrapie (Jeffrey and Gonzalez, 2007). The name is attributed to the clinical sign observed in sheep, consisting of repetitive scratching (Lampert et al., 1972). In addition to sheep the disease was also found in goats and mouflons (Jeffrey and Gonzalez, 2007).

In the 1960s chronic wasting disease (CWD) was discovered in North America (Williams and Young, 1980). This is the only known prion disease that occurs in wildlife, affecting elk, moose and deer (Haley and Hoover, 2015). The first cases of bovine spongiform encephalopathy (BSE) affecting cattle were described in the mid-1980s (Wells et al., 1987). It is not clear how the disease first appeared in cattle. The most accepted theory supports transmission through feeding the cattle with prion infected meat and bone (Weissmann and Aguzzi, 1997). This disease variant also named "mad cow disease", caused a lot of public attention because it was found to be transmissible to humans causing variant Creutzfeldt-Jakob disease (vCJD)

(Weissmann and Aguzzi, 1997). To reduce the spread of BSE many affected animals were killed and more stringent food controls were implemented. 30 years after the initial disease discovery, BSE is only rarely diagnosed (http://www.oie.int/en/animal-health-in-the-world/bse-specific-data/number-of-reported-cases-worldwide-excluding-the-united-kingdom/; http://www.oie.int/en/animal-health-in-the-world/bse-specific-data/annual-incidence-rate/).

4.1.2. Prion disease in humans

Amongst prion diseases in human, sporadic CJD (sCJD) is the most common form (Aguzzi, Calella, 2009). In most cases of sCJD, the affected persons are 60-65 years old but the disease can occur in teenagers and more elderly people as well (Kovacs and Budka, 2009). After diagnosis of the disease, patients typically die within two years, or in most cases even after a few months due to the rapid progression of dementia associated with ataxia and mycoclonus (Kovacs and Budka, 2009).

Genetic prion diseases are the next most frequent. Given that genetic prion diseases are caused by a mutation in the *PRNP* gene, it was suspected that somatic mutations in the *PRNP* locus occur also in sCJD (Prusiner, 1989). Furthermore it was suggested that the spontaneous conversion of PrP^{C} to PrP^{Sc} (Aguzzi et al., 2008a; Colby and Prusiner, 2011) or that infections are responsible for acquiring of sCJD (Aguzzi and Calella, 2009). However, none of these hypotheses could have been proven and the etiology of sCJD remains enigmatic (Aguzzi et al., 2008a; Aguzzi and Calella, 2009).

Prion diseases that are transmitted by direct contact with infectious material, including Kuru, iatrogenic and vCJD, are the least frequent form of prion diseases (Aguzzi and Calella, 2009). In the 1950s the first cases of Kuru in Papua New Guinea were reported (Gajdusek and Zigas, 1959, 1961). Kuru is characterized by cerebellar ataxia associated with tremor, choreiform and athetoid movements (Liberski et al., 2012). It was presumed that the disease was caused by the ritual consumption of tissue from dead relatives affected with sporadic prion disease, a common practice of aborigines to show honor their death relatives (Alpers and Gajdusek, 1965; Collinge et al., 2006).

vCJD is supposed to be caused by consumption of BSE affected cows (Aguzzi and Heikenwalder, 2006; Aguzzi and Calella, 2009). While direct experimental confirmation is missing, transmission of BSE to monkey and cows, epidemiological

and clinical and pathological studies as well as molecular biological experiments support this hypothesis (Aguzzi, 1996; Aguzzi and Weissmann, 1996; Bruce et al., 1997; Hill et al., 1997). Similar to sCJD, the disease progression is rapid (Zeidler et al., 1997).

Iatrogenic CJD is the most frequent form of prion diseases caused by contact with infectious material (Aguzzi and Calella, 2009). The transmission occurs accidently during medical procedures with patient material from affected donors or instruments that were before in contact with patients affected by prion disease. Documented examples include medical procedures including transplantation of dura mater, blood transfusions or positioning of stereotactic electrodes in the brain (Will, 2003).

Between 5 and 15 % of human prion diseases are a result of dominant autosomal mutations in the *PRNP* gene (Aguzzi et al., 2008b; Aguzzi and Calella, 2009). The pathological features, onset of disease and the speed in disease progression are dependent on alterations in the *PRNP* gene (Aguzzi et al., 2008b). In 1936 the Gerstmann-Sträussler-Scheinker (GSS) disease was first described by Josef Gerstmann, Ernst Sträussler, and Ilya Mark Scheinker (Zeidman et al., 2014). The disease is caused by mutations at codons 102, 105, 117, 145, 198 and 217 in the *PRNP* open reading frame (Tateishi et al., 1988; Hsiao et al., 1989; Ghetti et al., 1995; Aguzzi et al., 2008a). Compared to CJD, disease progression is rather slow (2-10 years) with chronic progressive dementia and dementia occurring at the final stages of the disease (Aguzzi et al., 2008b). 1986 Fatal Familial insomnia (FFI) was discovered in an Italian family (Lugares, 1986). The disease is caused by a D178N point mutation in the prion gene and methionine-valine polymorphism at codon 129 (Goldfarb et al., 1992). Characteristic features of the disease are sleep disturbance and diminished attention (Collins et al., 2001).

Interestingly, carriers of the single nucleotide polymorphism (SNP) (M129V) at codon 129 of *PRNP* are more susceptible to prion disease (Mitrova et al., 2005). In addition, this SNP influences the onset of disease (Poulter et al., 1992), disease progression (Mitrova and Belay, 2002) and clinical manifestation (MacDonald et al., 1996; Kovacs et al., 2000).

4.2. Prion mediated toxicity

Despite decades of prion research the mechanism underlying prion toxicity remains elusive. A neuroprotective function of PrP^C has ben postulated by many independent studies (Roucou et al., 2004; Roucou and LeBlanc, 2005) suggesting that loss of

function causes neuronal cell death in prion disease (Hetz et al., 2003; Westergard et al., 2007). However, neither pre- nor postnatal ablation of PrP^C has yielded any phenotypical prion pathology (Bueler et al., 1993; Mallucci et al., 2002) which leads to the assumption that loss of PrP^C function in prion disease is rather unlikely (Aguzzi et al., 2008a). Alternatively, it is possible that the role of PrP^C is only revealed under cellular stress and redundant under normal biological conditions (Harris and True, 2006).

Nevertheless experiments with PrP^C knock out mice showed that PrP^C is essential for prion mediated toxicity (Bueler et al., 1993; Weissmann et al., 1994). This result was confirmed by the fact that ablation of PrP^C in early stages of prion infected mice could reverse spongiform changes, prohibit neuronal loss and progression to clinical stages of the disease (Mallucci et al., 2003). The engraftment of PrP^C overexpressing tissue in the brain of PrP^C knock out mice was followed by inoculation with PrP^{Sc} (Brandner et al., 1996b; Brandner et al., 1996a). In PrP^C overexpressing tissue, replication of PrP^{Sc} as well as characteristic neuropathological changes of prion infection was reported (Brandner et al., 1996b; Brandner et al., 1996a). However, despite sufficient migration of PrP^{Sc} in PrP^C depletion regions no histopathological changes were seen in PrP^C deficient regions (Brandner et al., 1996b; Brandner et al., 1996a). Mice expressing PrP^C mutants lacking the GPI (glycosylphosphatidylinositol)-anchor, which directs PrP^C to the cell membrane, were able to replicate PrP^{Sc} but showed no signs of neurodegeneration (Chesebro et al., 2005). This lead to the conclusion that not only PrP^C expression but also its cellular localization is crucial for of neuronal loss (Chesebro et al., 2005).

So far the exact molecular mechanism underlying neurodegeneration in prion disease remains puzzling (Aguzzi et al., 2008b; Chiesa, 2015). One theory suggests that the prion protein itself is able to form a pore or interacts with cationic ion channels which leads to changes in the membrane potential causing cell death (Aguzzi and Falsig, 2012; Biasini et al., 2012). In particular it was observed that PrP^C mutants lacking the central region or containing point mutations, associated with familiar prion disease, induced spontaneous currents in several cell lines (Solomon et al., 2010). This effect could be rescued via overexpression of full length WT PrP^C (Solomon et al., 2010). Further evidence that ion channels might be involved in prion toxicity were provided by studies in PG14 mice (Tg) (PG14) mice (Senatore et al., 2012) that contain octapeptide insertions related to a genetic prion disease in

humans (Chiesa et al., 1998). In this mouse line, the association of mutant PrP^C with the voltage gated calcium channel α2δ-1 subunit leads to impaired neurotransmission, especially in cerebellar granule neurons (Senatore et al., 2012).
In addition it is proposed that impairment in the UPR elicits the neuronal loss of prion disease (Moreno et al., 2012; Moreno et al., 2013). This will be explained in more detail in chapter 6.3.2.

4.3. Structure of the cellular prion protein

The prion protein is a membrane anchored glycoprotein (Paulick and Bertozzi, 2008) encoded by the *PRNP* gene, which has a highly conserved structure amongst different mammalian species (Basler et al., 1986).
It includes an N-terminal flexible region, a globular domain containing three α-helices and a region with two antiparallel β-sheets that surround the globular domain (Riek et al., 1997; Zahn et al., 2000). The formation of a covalent disulfide linkage leads to the stabilization of the carboxy terminal region (Riek et al., 1996).
The prion protein can be divided in two large units, a N-terminal flexible tail and a C-terminal globular domain (Riek et al., 1997). The N-terminus consists of the signal peptide (SP), the small charge cluster one domain (CC1), the octapeptide region (OR), the charge cluster two domain (CC2) and the hydrophobic core (HC) (Aguzzi et al., 2008a; Aguzzi et al., 2008b). The SP is important for translation of PrP^C in the endoplasmic reticulum (ER), where it undergoes several post-translation modifications including glycosylation, disulfide-bond formation and finally cleavage of the SP, before trafficking via the Golgi to the cell surface (Harris, 2003). Since the OR is able to bind copper (Brown et al., 1997) many studies have suggested a protective effect of PrP^C against oxidative species (Steele et al., 2007). However, genetic ablation of PrP^C *in vivo* causes no significant impairment of SOD activity (Hutter et al., 2003). The HC seems to be involved in the stability of PrP^C because disease associated mutations in this region cause structural instability and misfolding of PrP^C (van der Kamp and Daggett, 2010). *In vitro* studies of HC deletion mutants suggest an involvement of the HC in prion propagation (Holscher et al., 1998; Norstrom and Mastrianni, 2005).
The α-helical structures within the GD undergo a drastic change to a beta-sheet rich structure during the conversion of PrP^C into PrP^{Sc} (Caughey et al., 1991; Pan et al., 1993; Safar et al., 1993). However, the physiological function of the GD is not well characterized (Ciric and Rezaei, 2015).

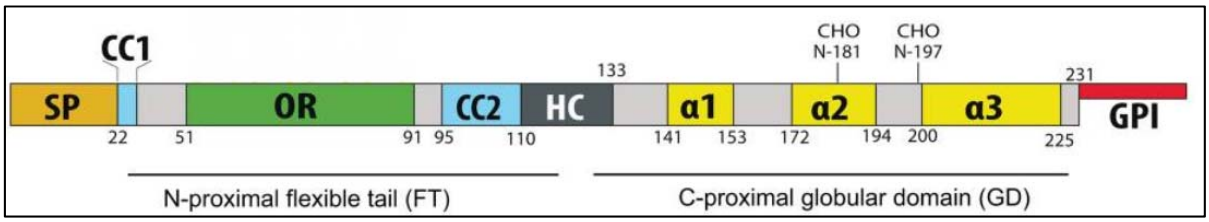


Figure 2: Primary structure PrP^C

The sequence of the PrP^C primary structure contains the following consecutive domains from the N- to the C-terminus: A secretory signal peptide (SP, orange), small charge cluster one (CC1, blue) the octapeptide repeat (OR, green), charge cluster two (CC2, blue), hydrophobic core (HC, dark grey), globular domain containing α1-, α2- and α3-helixes (yellow) (Dametto et al., 2015). In the above image, the following post-translational modifications are listed: glycosyl phosphatidyl inositol (GPI); facultative glycosylation sites (CHO) (Dametto et al., 2015) . The region of the domains is defined by the number of respective amino acids (Dametto et al., 2015). [figure modified (Dametto et al., 2015)]

5. Investigation of the role of glial cells in prion toxicity

5.1. Non-cell autonomous neurodegeneration

Interestingly, most cell lines are resistant to prion infection (Bosque and Prusiner, 2000), with only a few capable of to replicating prions, such as murine hypothalamic cell lines (Schatzl et al., 1997; Milhavet et al., 2000), the murine neuroblastoma cell line N2a (Race et al., 1987; Butler et al., 1988) and the rat pheochromocytoma cell line PC12 (Rubenstein et al., 1984). Nevertheless, none of these cell lines displayed neurotoxic effects as a result of prion infection (Vilette, 2008). Only hypothalamic GT1 cell lines have been shown to exhibit slight changes in cell viability (Schatzl et al., 1997; Milhavet et al., 2000).

In primary neurons prion mediated toxicity was triggered via direct treatment with PrP^{Sc} infected brain homogenate (Cronier et al., 2004), prion-infected astrocytes replicating PrP^{Sc} (Cronier et al., 2012), purified PrP^{Sc} preparations (Muller et al., 1993), specific peptides containing parts of the prion protein sequence (Forloni et al., 1993) and by expression of PrP^{C} deletion mutants (Watts et al., 2007). However, in all of these experiments, the absence of glia in neuronal cultures could not be confirmed, as all these models solely exploited glial-neuronal co-cultures (Forloni et al., 1993; Muller et al., 1993; Cronier et al., 2004; Cronier et al., 2012). Therefore, glial cells seem to be essential for prion-mediated neurotoxicity.

In the past, the pathology of neurodegenerative diseases was mainly considered to occur in a cell autonomous manner, meaning that damage in neurons is sufficient to cause disease without the contribution of another cell type (Ilieva et al., 2009). This view has changed due to evidence that neuronal cell death is influenced by mutant protein expression or toxicity in non-neuronal cell types in the vicinity of affected neurons (Lobsiger and Cleveland, 2007). In particular glial cells seem to be involved in neurodegeneration, as is the case in amyotrophic lateral sclerosis (ALS), spinocerebellar ataxia, Huntington's disease, and Parkinson's disease (Lobsiger and Cleveland, 2007).

It has been observed that astrocytes are involved in various cellular processes including the regulation of blood flow (Howarth, 2014; MacVicar and Newman, 2015), blood brain barrier integrity (Abbott et al., 2006), brain energy homeostasis (Belanger et al., 2011) and control of synapse formation and function (Clarke and Barres, 2013). It has been shown that mis-regulation of some of these processes leads to neuropathological changes (astrocyte–neuron interactions in neurological disorders,

Ricci, 2009). For example, in Alzheimer's disease it has been suggested that the blood brain barrier is impaired through a malfunction of astrocyte resident receptors responsible for shuttling of amyloid β (Aβ) from and into the brain [140, 141]. In addition it gas been proposed that Aβ changes calcium signaling of astrocytes and thereby may modulate synaptic plasticity [143].

In prion disease, the accumulation of PrP^{Sc} takes place in astrocytes during early disease stages (Diedrich et al., 1991) with significant morphological abnormalities in astrocytes observed during disease progression (Eklund et al., 1967). The observation that expression of astrocyte PrP^{C} is sufficient to induce susceptibility to prion disease (Raeber et al., 1997; Jeffrey et al., 2004) is supported by the concept that astrocytes could be the non-cell autonomous component in prion disease. However, these data is contradicted by the observation that depletion of PrP^{C} in mouse models with early prion disease could reverse spongiform changes and prevent neuronal loss (Mallucci et al., 2003).

5.2. *In vivo* and *ex vivo* models of prion disease

Mice and hamsters represents the most frequent animal models for studying prion disease (Groschup and Buschmann, 2008; Watts and Prusiner, 2014). The advantage of hamsters is that they possess a very short incubation time before displaying a pathological phenotype (Kimberlin and Walker, 1977). However, for most *in vivo* prion studies mice are preferred over hamsters because they are easier to handle and this model offers superior genetic manipulation options (Watts and Prusiner, 2014). Disease progression in mouse models can be accelerated through overexpression of PrP^{C} (Raeber et al., 1998). Nevertheless, the experiments remain time-consuming, expensive and the pharmacological manipulation of these rodent models are not very efficient (Doke and Dhawale, 2015). A great advance in the prion field was the invention of prion organotypic slice culture assays (POSCA) (Falsig et al., 2008), which closely simulate intracerebral infections with prions including characteristic features like prion propagation, inflammation, spongiform changes and neuronal loss (Falsig et al., 2012). Nevertheless, the slice culture system is complex which makes it challenging to discriminate between the effects from different cell types. Therefore it might be preferable to study cell-autonomous and non-cell autonomous mechanisms in a mono- or co-culture system. Cell culture systems have the advantage of being more suitable for experimental manipulations such as RNA

interference, calcium imaging and cell viability assays. Additionally, slice cultures are time consuming to prepare and not suitable for high-throughput screening. However, as mentioned under 5.1, prion toxicity could not be successfully reproduced in cell lines or pure primary neuronal cultures (Vilette, 2008). Prion mediated toxicity could only be found in primary neuron-glial co-cultures (Cronier et al., 2012; Cronier et al., 2004; Forloni et al., 1993; Muller et al., 1993). Therefore, primary neurons in co-culture with glial cells seem to be suitable for a prion *in vitro* model.

5.3. Antibodies mimicking prion toxicity

Recently it has been shown that antibodies targeting the globular domain of PrP^C like POM1, elicited neuronal cell loss in cultured organotypic cerebellar slices, which appears to be mediated by pathways similar to prion infection (Sonati et al., 2013; Herrmann et al., 2015). In addition, the toxic effect of POM1 depends on expression levels of PrP^C, as is also the case in prion infections (Sonati et al., 2013). This system represents a non-infectious model for prion disease with faster kinetics and allows us to focus on the toxic pathways involved in disease (Sonati et al., 2013; Herrmann et al., 2015).

5.4. Scientific Aims

One of the major aims of the thesis was to establish a cell culture model with the help of POM1 that reflects prion toxicity. This included the following steps:

- It has already been shown that in primary neurons prion toxicity was mediated by the presence of glial cells (Cronier et al., 2012; Cronier et al., 2004; Forloni et al., 1993; Muller et al., 1993).
- Therefore the potential of astrocytes and microglia to render primary neurons vulnerable to POM1 mediated toxicity was explored.
- A suitable detection system was established for measuring POM1 mediated cell death *in vitro*.
- POM1 mediated neuronal loss was assessed with the selected detection system.

5.5. Results

5.5.1. Detection of POM1 mediated toxicity in a pure neuronal mono culture system

5.5.1.1. Cell viability assays could not reveal any POM1 induced toxicity

It has been proposed that disease progression in prion disease can be split into two phases: in the first phase prions replicate until they reach a plateau and in the second phase the clinical signs of the disease start to manifest (Sandberg et al., 2011; Aguzzi and Falsig, 2012). To establish a cell culture model that replicates prion toxicity we used the anti-PrP^C antibody POM1. POM1 is an antibody targeting the globular domain of PrP^C (Polymenidou et al., 2008) and has been shown to mimic prion mediated toxicity in cerebellar organotypic slice cultures (Sonati et al., 2013; Herrmann et al., 2015). We selected this toxicity model over the use of prion infected brain homogenate for the following reasons:

POM1 mediated toxicity and cell death is faster and only reflects the neurotoxic and not the inflammatory component of disease progression (Sonati et al., 2013; Herrmann et al., 2015). POM1 induced molecular changes can therefore be specifically attributed to toxicity.

Furthermore, the antibody is considered a level 1 biosafety agent, which can be used for the design of easy-to-handle, infectivity-free model systems of prion toxicity (Sonati et al., 2013; Herrmann et al., 2015).

The *in vitro* model system was initially analyzed by two former lab members; Dr. Tracy O'Connor and Sine Yaganoglu; POM1 was applied to primary cortical neuronal cultures supplemented with astrocyte media or astrocyte conditioned media collected from PrP^C overexpressing (*tga20)* or PrP^C knock out (ZH1) astrocytes. They observed no toxicity in pure cortical neuron cultures from *tga20* mice overexpressing PrP^C in astrocyte media, whereas variable results were obtained when neurons were grown in astrocyte-conditioned media. Neuronal loss was evaluated with the Lactate-Dehydrogenase (LDH) Assay: cell death leads to the loss of the cell membrane integrity and LDH is released into the media (Korzeniewski and Callewaert, 1983; Decker and Lohmann-Matthes, 1988). LDH is quantified by its ability to reduce NAD^+ to NADH (nicotinamide adenine dinucleotide), which then converts the tetrazolium salt Iodonitrotetrazolium to formazan in a colorimetric reaction (Korzeniewski and Callewaert, 1983; Decker and Lohmann-Matthes, 1988). The main limitation of the LDH assay is its dependence on the enzymatic activity of LDH, an enzyme prone to degradation, easily influenced by pH and compound/treatment condition changes in

the cell culture media (Galluzzi et al., 2009). Due to the unreliable readout of the LDH assay, the dependence of POM1 induced neurotoxicity in primary cortical neurons on astrocyte-secreted factors remains elusive.

To further investigate this question, I[1] tested additional cell viability/cytotoxicity assays to assess POM1 mediated toxicity *in vitro*. The following assays were selected: LIVE/DEAD assay, Cell Titer Glo and alamarBlue.

Due to its robustness, easy and quick readout, the alamarBlue assay was chosen for further studies. The activate agent of the assay is resazurin, a nontoxic, cell permeable dye. In the reducing environment of a viable cell the compound is converted to resorufin, which exhibits a bright red fluorescence and represents a quantitative measure of cell viability (Nociari et al., 1998; Rampersad, 2012). To assess POM1 mediated toxicity *in vitro,* primary cortical neurons derived from *tga20* mice were plated in a 96-well plate. After 5 days the cells were treated with POM1 in astrocyte media (AM) or astrocyte conditioned media (ACM) obtained from *tga20* astrocytes. Recombinant prion protein (rPrP) was used to inhibit the effect of POM1. 6h before the measurement, alamarBlue was added to neuronal cultures and endpoint readings were done at 24h and 48 h after addition of POM1. The applied concentration of 100 nM POM1 caused widespread neuronal death in cultured organotypic slices from *tga20* pups (Sonati et al., 2013; Herrmann et al., 2015), yet the same concentration did not induce toxicity in cortical neurons cultured in AM or ACM. In contrast, the positive control Staurosporine (ST), a well-documented agent of apoptosis (Tamaoki et al., 1986; Kruman et al., 1998; Belmokhtar et al., 2001), led to decreased cell viability, confirming the validity of the assay.

1 Based on the suggestion of my thesis supervisor Prof. Dr. Adriano Aguzzi I elected to use the first-person narrative to make an unambiguous assignment to the experiments I have performed. For experiments where other persons where involved in planning, analysis and/or interpretation of the data I have chosen the “we” designation or particular mentioned the person who was involved in the experiment.

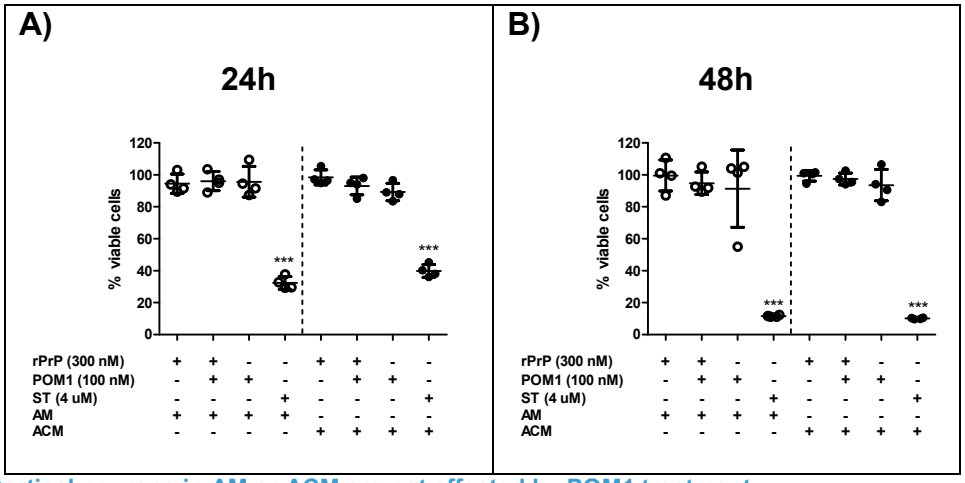


Figure 3: Cortical neurons in AM or ACM are not affected by POM1 treatment
5 DIV (days in vivo) t*ga20* primary cortical neurons were treated with rPrP (300 nM), POM1 (100 nM) plus rPrP (600 nM) or ST (4 uM) in presence of AM or ACM. The cell viability of the neurons was assessed with the alamarBlue assay 24 h (A) or 48 h (B) after treatment. *Each error bars indicate standard deviation (SD) of 3 technical replicates. A one-way ANOVA with Dunnett's post-test was used for this experiment and for all multiple comparisons (p < 0.005 = ***)*

5.5.2. Using molecular markers to evaluate POM1 mediated toxicity in cell culture

5.5.2.1. Evaluation of transcriptional changes in POM1 treated primary cortical neurons

Immediate Early Genes (IEG) are rapidly and transiently activated in response to a cellular stimulus (Kubik et al., 2007; Perez-Cadahia et al., 2011). In the brain they are involved in processes like neuronal plasticity and learning, suggesting that they might also be activated during the early stages of neurodegeneration (Qiao et al., 2007; Loebrich and Nedivi, 2009; Koldamova et al., 2014). In order to assess the role of IEG in POM1 toxicity, mRNA expression of specific IEG following POM1 treatment was quantified by qRT-PCR (quantitate real time polymerase chain reaction).

I used 5 day old cortical neurons, treated with rPrP (600 nM), POM1 (200 nM) plus rPrP (600 nM), POM1 (200 nM) or ST (4 um) in ACM. After 1h incubation cells were lysed and collected for mRNA isolation. I initially identified a set of housekeeping genes with minimal variation in the experimental setup used here (Vandesompele et al., 2002). To proceed, I investigated the expression of housekeeping genes involved in different cellular pathways (see Table 1) across 12 samples (see Figure 4).

Table 1: Housekeeping genes for qRT-PCR

Gene	Function	Reference
B2m	Histocompatibility	(Wang et al., 2010a)
elF2α	Protein translation	(Kosir et al., 2010)
GAPDH	Glicolysis and gluconeogenesis	(Seidler, 2013)
Gusb	Carbohydrate metabolism	(Wang et al., 2010a)
Hmbs	Heme synthesis, porphyrin metabolism	(Kosir et al., 2010)
Ppib	secretory pathway	(Kosir et al., 2010)
Utp6c	Rn18 s biogenesis	(Kosir et al., 2010)
Tbp	protein peptidyl-prolyl isomerization	(Wang et al., 2010a)

The qRT-PCR data was analyzed with a Visual Basic Application (VBA) for Microsoft Excel called GeNorm (Vandesompele et al., 2002). This application computes the gene-stability measure M for a selected gene by evaluation of the variation for all other control genes used (Vandesompele et al., 2002). The lowest M value, and the therefore lowest variability, was observed for elF2α, Htp6c and Ppib, which were therefore chosen for the normalization of my qRT-PCR data (see Figure 4). Interestingly, the M value for GAPDH was quite high compared to the selected genes. However, in many qRT-PCR protocols, this gene is used as reference (de Jonge et al., 2007; Kozera and Rapacz, 2013). This highlights the importance of selecting appropriate normalization controls to decrease the variability of results.

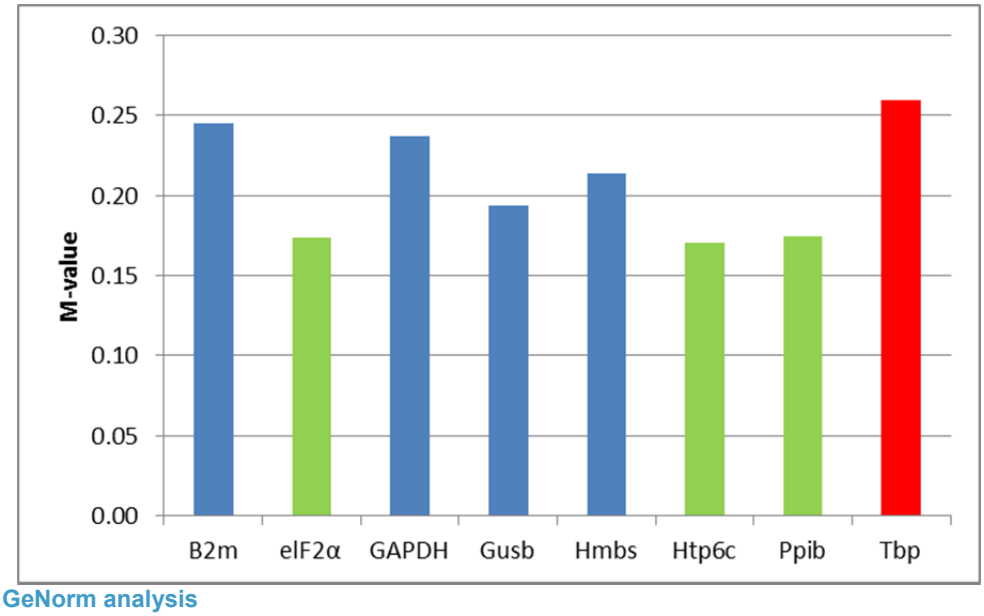


Figure 4: GeNorm analysis

qRT-PCR was done with isolated RNA from 1h with POM1 (200 nM), POM1 (200 nM) plus rPrP (600 nM) or ST (4 uM) treated and untreated cortical neurons in ACM (technical replicates RNA isolation n = 3; technical replicates qRT-PCR n=3). The M-Value was calculated with GeNorm, a Visual Basic Application (VBA) for Microsoft Excel. The genes with the lowest M-values are the most stable expressed ones. Therefore elF2α, Htp6c and Ppib were selected for normalization of the actual experiment.

I next decided on a set of IEGs to define early changes post POM1 treatment: Arc, CREB (cAMP response element-binding protein), c-Jun, Egr1 (Early growth response protein 1) and Homer1a. The transcription factor Egr1 is downregulated in prion disease (Booth et al., 2004; Sorensen et al., 2008) and in APP/PS1 Alzheimer's disease animal models (Dickey et al., 2003; Dickey et al., 2004). The postsynaptic protein Arc is a downstream target of Egr1 (Koldamova et al., 2014) and involved in synaptic plasticity (Knapska and Kaczmarek, 2004; Kubik et al., 2007). In the Alzheimer mouse model, *APPswe;PS1ΔE9,* the depletion of Arc prevents an activity dependent generation of Aβ suggesting an involvement or Arc in Alzheimer's disease pathology (Wu et al., 2011). The transcription factor c-Jun is upregulated in primary cortical neurons in response to prion infection and treatment with the toxic human PrP^C peptide PrP106-126 (Carimalo et al., 2005), and increases in cerebellar brain slices upon POM1 treatment (Herrmann et al., 2015). The (CaMK4β)/CREB signaling is misregulated in prion infected mice (Sorensen et al., 2008; Shott et al., 2014). The scaffold protein Homer1a is induced upon synaptic activity (Brakeman et al., 1997; Kato et al., 1998) and downregulated in an Alzheimer's disease animal model (Dickey et al., 2003). Homer1a has also been reported to bind to metabotropic glutamate receptors (Brakeman et al., 1997), which have been shown to be bound by PrP^C (Um et al., 2012; Um and Strittmatter, 2013). These reports suggest that Arc, CREB, c-Jun, Egr1 and Homer1a might also be affected during prion-induced toxicity, assessed by POM1 treatment.

Cortical neurons in ACM were cultured and treated with POM1 (200nM) for 1h, neurons were collected and RNA was extracted as mentioned above. Expression of the previously mentioned IEGs after 1h treatment with POM1 was evaluated with qRT-PCR, yet none of the investigated genes significantly changed upon POM1 treatment (see Figure 5).

This observation could be due to several reasons. The selected genes are not affected after POM1 treatment or transcriptional changes occur in another time frame. As mentioned above astrocyte-secreted factors might be required for POM1 mediated toxicity, and therefore also be required for the activation of IEG. We therefore decided to use an astrocyte-neuron co-culture for future experiments.

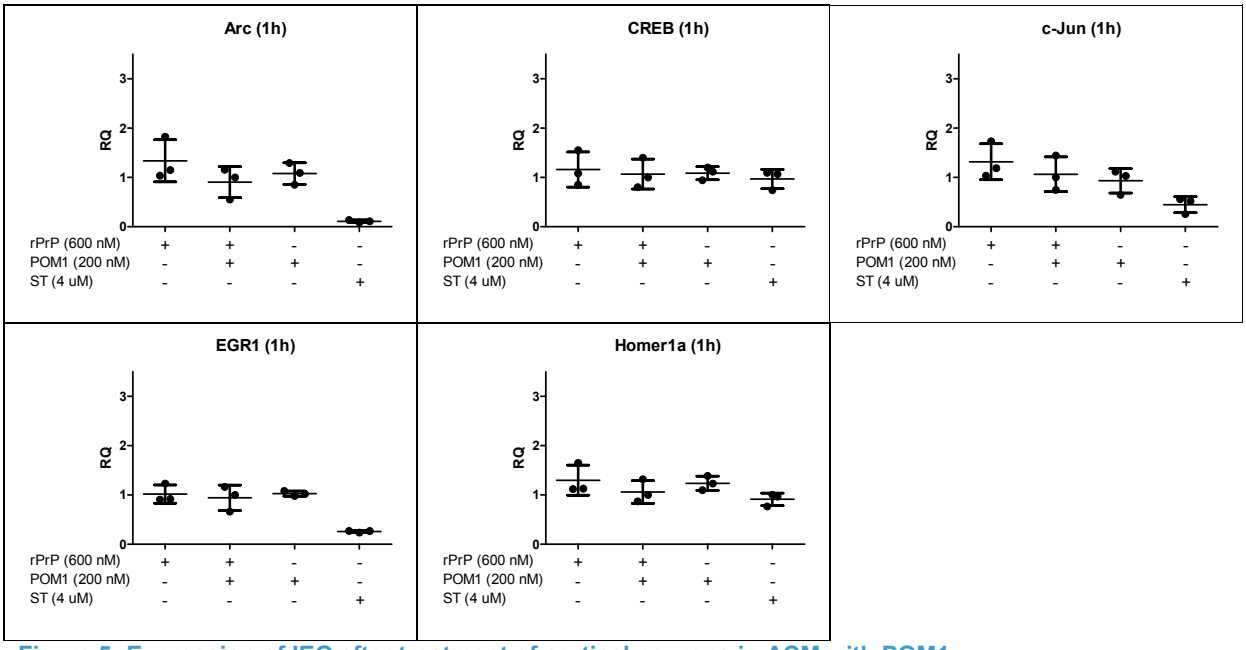


Figure 5: Expression of IEG after treatment of cortical neurons in ACM with POM1

5 DIV cortical neurons were treated with POM1 (200 nM), POM1 (200 nM) plus rPrP (600 nM) or ST (4 uM). After 1h incubation time the cells were lysed and collected for RNA isolation. After purification and reverse transcription of the mRNA, qRT-PCR was performed. The Relative Quantities (RQ) are based on the normalized to the house keeping genes pelF2α, Htp6c and Ppib. *Each error bar indicates SD of 3 separate wells of cortical neurons analyzed (3 technical replicates for qRT-PCR were used = one dot). A one-way ANOVA with Dunnett's post-test was used for this experiment and for all multiple comparisons.*

5.5.3. Detection of POM1 mediated toxicity in neuron astrocyte co-culture system

5.5.3.1. Live cell imaging of POM1 treated neuron astrocyte co- culture systems

The alamarBlue Assay is a useful tool to evaluate cell viability in a pure cell culture system but it cannot discriminate between cell viability of different cell types. In addition, only an endpoint measurement is possible, which makes it difficult to acquire information regarding behavior of the cell during treatment. Therefore, we decided to use live cell imaging as a read out for the POM1 treatment of astrocyte-neuron co-cultures.

To discriminate neurons from astrocytes fluorescently labeled neurons were used. Primary neurons from *tga20* mice, overexpression PrP^C, are not fluorescently tagged. As an alternative we derived neurons from mouse embryonic stem cells (mESCs) expressing a neuron-specific fluorescent marker. This assay has two significant advantages: (1) experiments can be better coordinated, since it is not necessary to rely on the mice estrous cycle of the animals, (2) derivation of neurons form mESCs

follows a strict protocol thereby reducing the likelihood for experimental inconsistencies.

In particular, we decided to use two different mESC lines:

- NT mESC line (Di Giorgio et al., 2007) for derivation of moto- and interneurons
- TK-23 mESC line (Tucker et al., 2001) for derivation of a pan-neuronal culture

For derivation of mESCs into moto- and interneurons I utilized a modified protocol (Wichterle et al., 2002), provided by my supervisor Dr. Vijay Chandrasekar (see Figure 6). Specific derivation of mESCs into moto- and interneurons was based on the addition of the following factors: retinoic acid (RA) and Smoothed agonist (SAG) (Di Giorgio et al., 2007). Further enrichment of a moto- and interneuron specific population was achieved via expression of GFP under the control of a moto- and interneuron specific promotor, the homeodomain transcription factor 9 (HB9) (Tanabe et al., 1998; Arber et al., 1999; Wilson et al., 2005). This leads to a bright expression of GFP in moto- and interneurons, which enables gating and enrichment of these cell populations by FACS (fluorescence-activated cell sorting) (see Figure 7). Selected cells were then directly sorted in with primary astrocyte-coated plates.

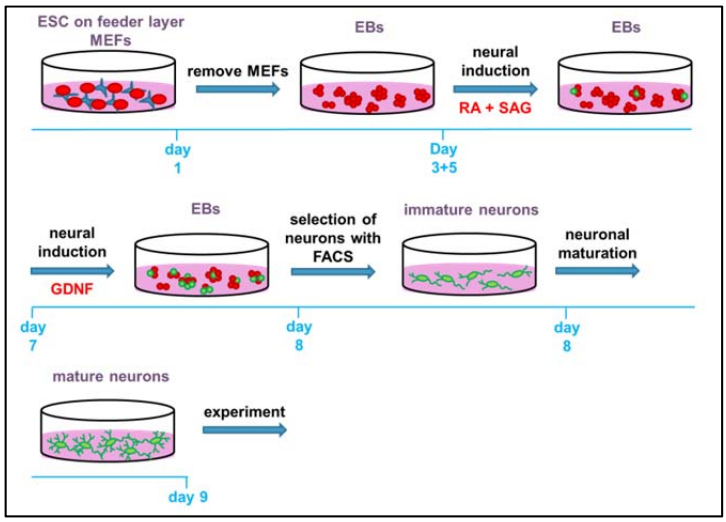


Figure 6: Protocol for derivation of moto- and interneurons for mESC
mESC were expanded on MEFs. On day 1 the cells were put into suspension culture were they aggregate and form structures named embryoid bodies (EBs). On day 3 and 5 neuronal differentiation was induced via RA and SAG. On day 7 the EBs were supported with the trophic factor GDNF (Glial cell-derived neurotrophic factor). The specific expression of GFP under a moto- and interneuron specific promotor enables the enrichment of the cells via FACS on day 8. After neuronal maturation for 1 day the experiment was started.

Motoneurons are not the main type of neurons affected in prion disease (Kovacs and Budka, 2009). Therefore, the generation of a pan-neuronal culture was needed to cover several types of neurons that could be affected in prion disease. For this purpose, the mESCs (TK23) were obtained from Austin Smith's group, United Kingdom (Tucker et al., 2001). For the derivation of a pan-neuronal linage, a suspension culture was used similar to that employed for moto- and interneuron generation. However, the induction of the neuronal linage was different. For the derivation of pan-neurons RA and Forskolin were added on days 5 and 7. On days 9, 11 and 13 the cells were supported with GDNF. The TK23 mESCs express EGFP under the neuron-specific promotor Tau (Binder et al., 1985; Tucker et al., 2001). This allowed the neuronal selection via FACS on day 14. Directed differentiation towards a neuronal lineage was based on a standardized protocol provided by Vijay Chandrasekar (Ying et al., 2003).

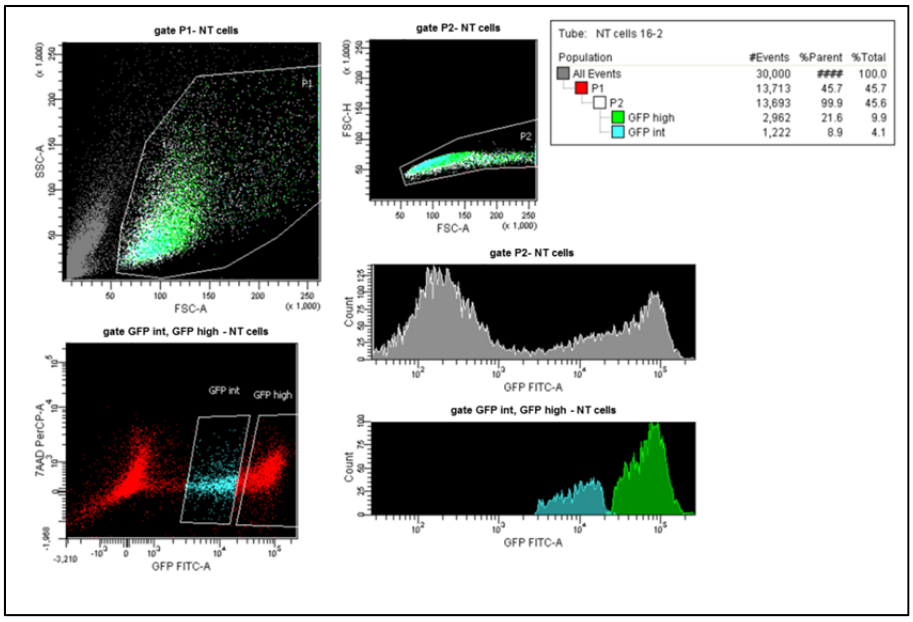


Figure 7: FACS example gating of NT mESC derived moto- and interneurons
The gate P1 was used to select viable cells. The forward scatter (FSC) roughly defines size and shape and the side scatter (SSC) reflects the complexity and granularity of the cells. The P1 gate was used to exclude apoptotic (low FSC and high SSC) as well as dead cells (low FSC and low SSC). The plot GFP FITC-A vs. 7ADD PerCP-A was used to select the viable cells (low 7ADD PerCP-A signal) with am intermediated GFP signal (interneurons progenitors, gate GFP int) and a high GFP signal (motoneurons progenitors, gate GFP high). The histogram of the gate P2 reflects the counts of the GFP positive cells while the histogram of the gate GFP int and GFP high reflects the events of the inter- and motoneurons progenitors.

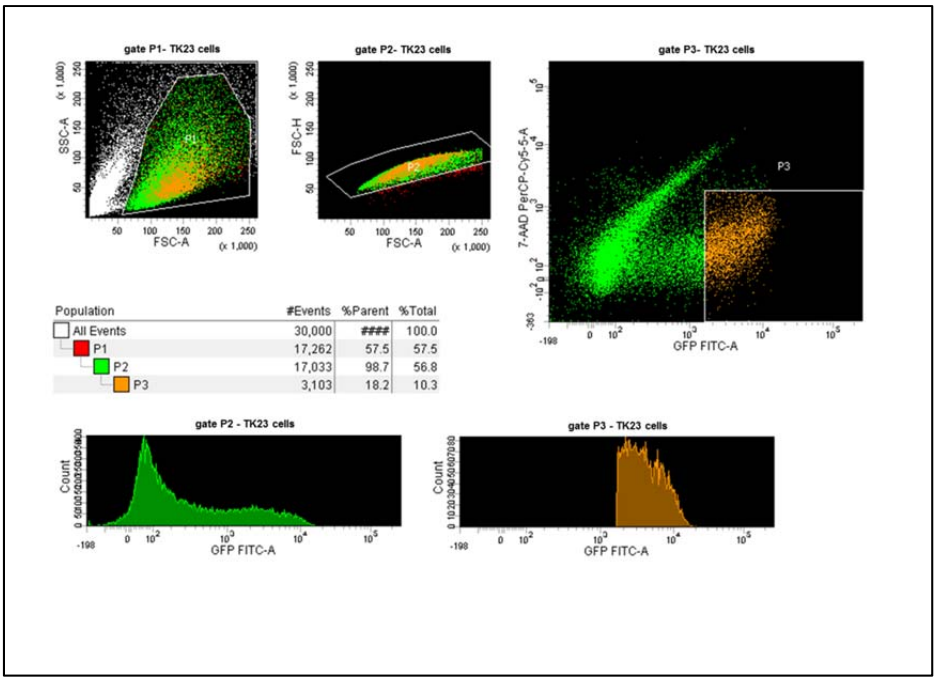


Figure 8: FACS example gating of TK23 mESC derived pan-neurons
The viable cells were selected with gate P1. The forward scatter (FSC) roughly defines size and shape and the side scatter (SSC) reflects the complexity and granularity of the cells. The P1 gate was used to exclude apoptotic (low FSC and high SSC) as well as dead cells (low FSC and low SSC). The plot GFP FITC-A vs. 7ADD PerCP-Cy5-5-A was used to select the viable cells (low 7ADD PerCP-A signal) with a high GFP signal (gate P3) and reflects the population of viable pan-neuronal progenitors. The histogram of the gate P2 reflects the counts of the GFP positive cells while the histogram of the gate P3 reflects the events of pan-neuronal progenitors.

Neuronal morphology is a general parameter indicating health status (M et al., 2015). For characterization of neuronal morphology, images were processed using the MetaMorph Neurite Outgrowth software package (Molecular Devices), which enables calculation of morphological changes based on multiple parameters, namely neurite outgrowth, number of processes and number of branches (see Figure 9).

Figure 9: Image processing with MetaMorph
A) and B) Live cell images were acquired with the Plate Runner, a fluorescence imaging plate reader (Trophos). C) The image processing was performed using the MetaMorph neurite outgrowth program (Molecular Devices) to quantify total neuronal number and their morphological changes. The software masks neurons (see image C) cyan) and calculates the number of cells displaying significant outgrowth, the mean neurite length/cell, the mean branches/cell and the mean processes/cell. These parameters are then used to define morphological changes after treatment.

5.5.3.2. Treatment of mouse moto- and interneuron astrocyte co-culture with POM1

Motoneurons from NT mESCs were generated as described in 5.5.3.1 and plated with primary *tga20* or Zurich 1 ($PrP^{-/-}$) astrocytes. One day after plating, a baseline reading was performed and the astrocyte neuron co-cultures were treated with POM1 (200 nM) or POM1 (200 nM) + rPrP (600 nM) in motoneuron media supplemented with GDNF and B27 minus antioxidants started. POM1 toxicity could be prevented by antioxidants (Sonati et al., 2013; Herrmann et al., 2015). Therefore, it was not used. Every other day the complete medium was exchanged and fresh POM1 was added. Neuron viability was evaluated with live cell imaging and morphological analysis of neurons was performed as stated in 5.5.3.1.

Motoneurons plated with PrP^{C} knock out or overexpressing astrocytes showed no significant changes in cells with significant neurite outgrowth (see Figure 10 A and C). At day 4 and 5 a decrease in the neurite outgrowth could be detected following POM1 plus rPrP treatment. This was somewhat surprising because rPrP was used to block the effect of POM1.

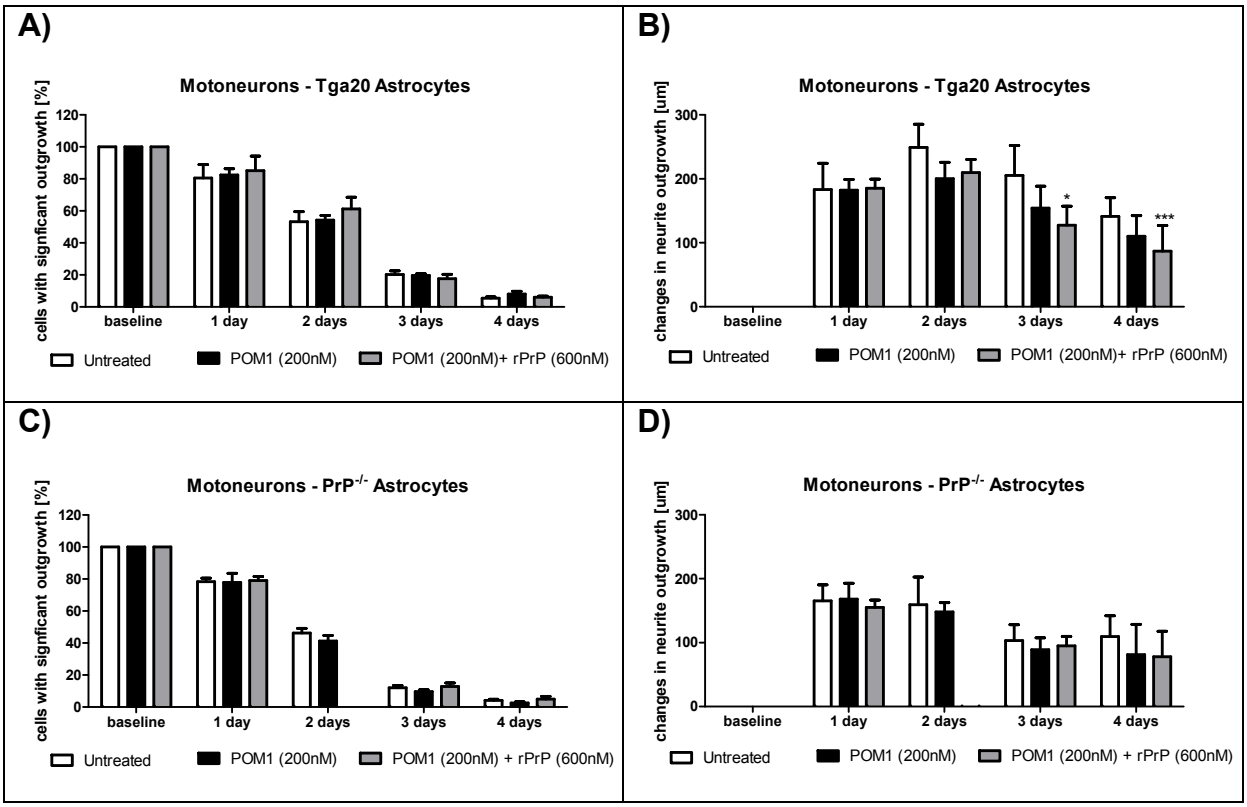


Figure 10: POM1 treatment does not induce toxicity in motoneurons astrocyte co-cultures
On day 1 NT mESC derived motoneurons, expressing wt PrP^C, were plated in co-culture with *tga20* astrocytes, overexpressing PrP^C or ZH1 astrocytes, deficient of PrP^C. From day 2 onwards cells were treated with POM1 (200 nM) or POM1 (200 nM) + rPrP (600 nM). To assess cell viability the number of cells with significant outgrowth and changes in neuronal outgrowth were assessed. Neither in motoneurons in co-culture with PrP^C expressing (A, B) nor PrP^C depleted astrocytes (C, D) a significant neuronal loss or morphological changes could be detected following POM1 treatment. *Each error bar indicates the SD of 5 (C, D) or 10 (A, B) technical replicates. A two-way ANOVA with a Bonferroni post hoc test was used for evaluation of this experiment whereby all samples were compared to untreated cells. Due to a reading error data points for POM1+rPrP at day 3 (C, D) are omitted. ($p < 0.001$ = ***, $p < 0.1$ = *)*

5.5.3.3. Treatment of mouse pan-neuron astrocyte co-culture with POM1

While it was reported that motoneurons could be affected in prion disease patients (Worrall et al., 2000), conflicting studies suggests that this population of neurons are not especially affected in prion disease (Budka, 2003). This could be the reason for the lack of any toxicity after POM1 treatment of motoneurons. Therefore, we decided to repeat the experiment in a pan-neuronal culture derived from TK23 mESCs. Derivation of neurons was performed as is described in 5.5.3.1. After FACS sorting neurons were plated in co-culture with *tga20* astrocytes. The absence of toxicity in the motoneuron co-cultures could indicate that the concentration of POM1 was insufficient to trigger neurodegeneration. Therefore, in this experiment several concentrations of POM1 were tested. In cerebellar slices from *tga20* mice, neuronal loss could be detected after a 3 day treatment with 67 nM POM1 (Sonati et al., 2013;

Herrmann et al., 2015). Furthermore, it has been shown that the level of POM1 toxicity is dose dependent (Sonati et al., 2013). Therefore, it was assumed that neuronal loss in the co-culture system could be observed if POM1 was used in a range from 50 nM to 250 nM. The advantage of TK23 derived pan-neurons is that they reflect a population of different types of neurons. However, compared to the NT mESC-derived motoneurons, TK23 mESC-derived pan-neurons show weak fluorescence. Thus only changes in cell number and not morphological differences in neurites could be evaluated with the MetaMorph software. None of the selected concentrations of POM1 induced notable toxicity within mESC-derived pan-neurons during the treatment period of 5 days (see Figure 11).

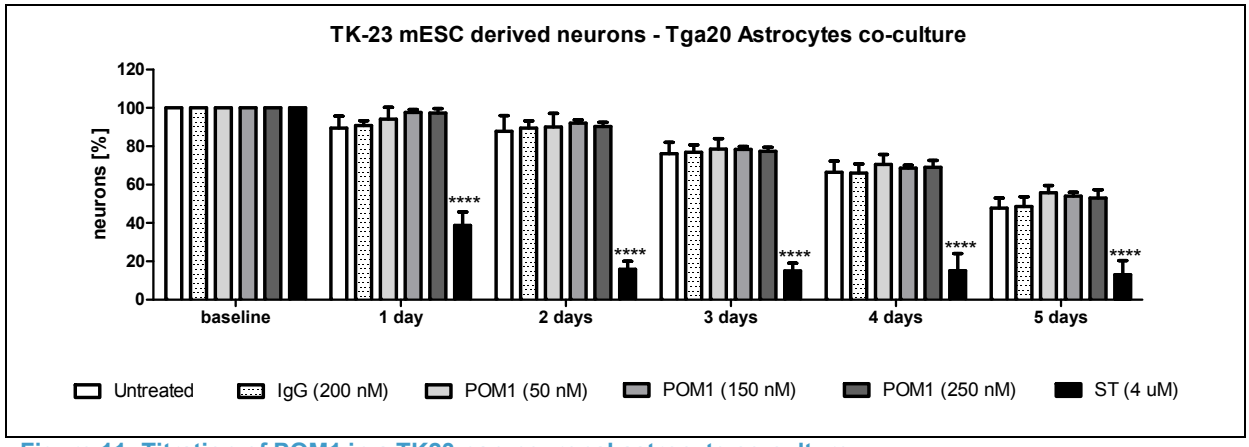


Figure 11: Titration of POM1 in a TK23 pan-neuronal astrocyte co-culture
TK23 mESC derived pan-neurons were co-cultured with primary *tga20* astrocytes. One day after plating (day 2) pan-neurons were treated with different concentration of POM1. Treatment with IgG (200 nM) or ST (4 uM) was used as a negative and positive control respectively. *Each error bar indicates average SD of 3-5 technical replicates (data points needs to be excluded because some images were overexposed and could not be properly analyzed by MetaMorph software). A two-way ANOVA with a Bonferroni post-hoc test was used for this experiment and all samples were compared to untreated cells. ($p < 0.001$ =****)*

5.5.4. Characterization of early molecular changes in human pan-neurons

Based on the cell viability assays, POM1 did not reveal any toxicity. Therefore molecular changes upon POM1 treatment were explored. To evaluate changes of POM1 in a human context, we opted to use induced pluripotent stem cells (iPSC) derived pan-neuronal cultures. Derivation of pan-neuronal cultures was performed according to a protocol from Dr. Vijay Chandrasekar. In brief iPSCs were dissociated and placed in suspension culture to promote aggregation of EBs. For differentiation into a pan-neuronal linage retinoic acid and GDNF were added. After a differentiation period of 20 days, neurons were seeded on pre-coated plates or coverslips.

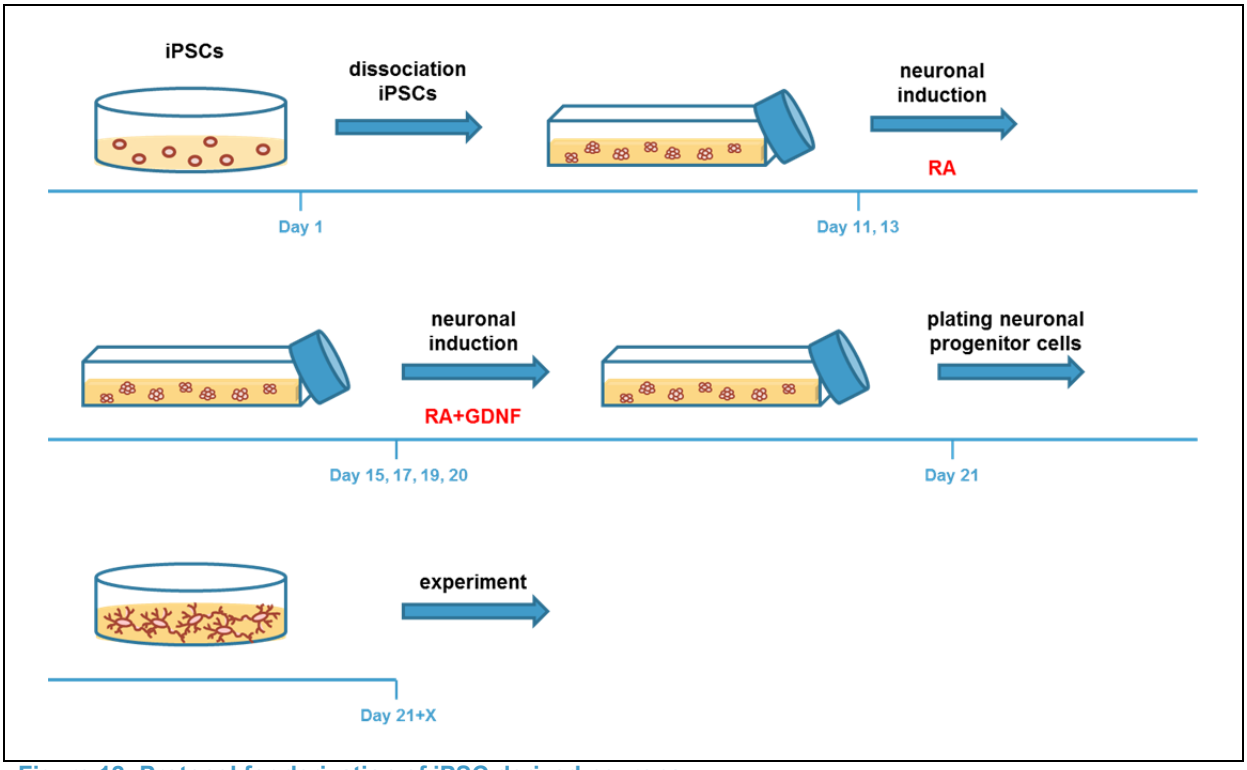


Figure 12: Protocol for derivation of iPSC derived neurons
iPSCs were amplified and put in suspension culture on day 1 for derivation of human neurons. On day 11, 13, 15, 17 and 19 the cells were driven towards a neuronal linage by addition of RA. From day 15 onwards the trophic factor GDNF was added. On day 21 the cells were digested and plated for the experiment.

Murine primary astrocytes from wildtype C57BL/6J mice were split into pre-coated cell inserts. After 3 days mouse primary astrocytes were put in a sandwich culture with wildtype human neurons to commence treatment with POM1 (400 nM). Media supplemented with fresh POM1 was exchanged every other day and. Since antioxidants have been implicated to illicit a neuroprotective function against POM1-mediated neuronal loss (Sonati et al., 2013; Herrmann et al., 2015), B-27 medium minus antioxidants was used during the whole antibody treatment. Since the cells are maintained in a sandwich culture, the neurons could be easily collected for western blot analysis after 7 days of treatment.

For biochemical analysis the proteins P-eIF2α, P-Erk1/2, P-Perk and Nur77 (NR4A1), were assessed because, they are known to be up regulated in POM1 and prion mediated toxicity (Herrmann et al., 2015). In addition, we checked the expression level of mGluR5, which has been implicated to interact with PrP^{C} in Alzheimer's Disease, thereby initiating neurodegeneration via the Fyn pathway (Um and Strittmatter, 2013). The levels of P-eIF2α and P-Erk1/2 were slightly increased upon POM1 treatment but an even higher upregulation could be observed in human

neurons treated with POM1 and rPrP (see Figure 13 A and B). Western blot analysis of P-Perk showed only a weak signal at the predicted size of 170 kDa (see Figure 13 C). This could be either due to a very low P-Perk expression level or perhaps to a technical problem while preforming the western blot. Levels of Nur77 show no obvious changes in the expression level (see Figure 14 A). However, upon POM1 and POM1 plus rPrP treatment, the lower band around 70 kDa was shifted to a slightly increased molecular weight. POM1 and POM1 plus rPrP treated cells notably also displayed a down regulation in mGluR5 (see Figure 14 B). In general no significant changes were observed and pre-incubation of POM1 with rPrP seems to further pronounce the slight effect seen following POM1 treatment. However, this was merely a preliminary study with only one sample per condition. Accordingly, more replicates are needed to interpret the data.

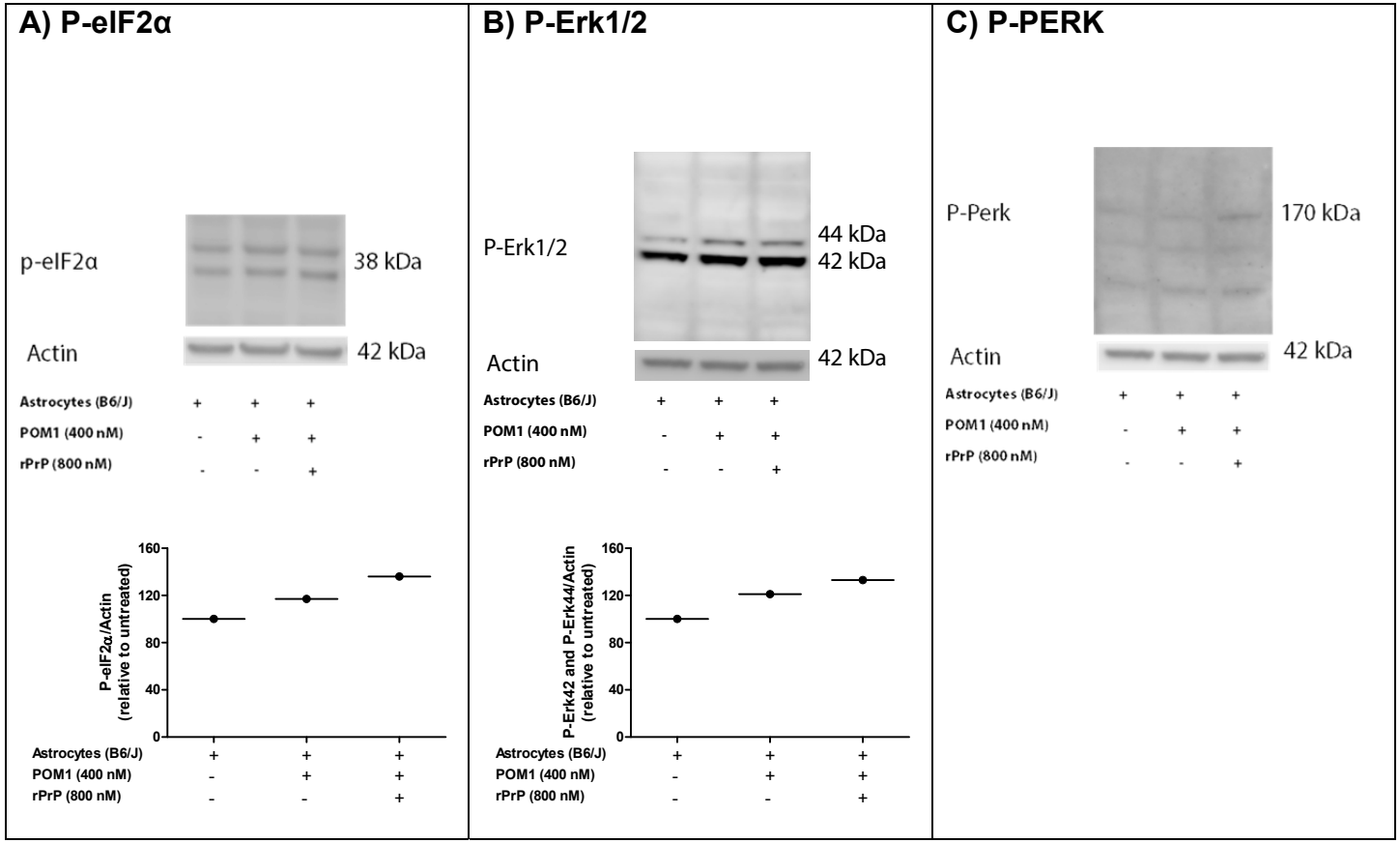


Figure 13: POM1 co-culture human neurons – mouse astrocytes

iPSC derived human neurons were co-cultured with primary mouse astrocytes and treated every other day with POM1 (400 nm) or POM1 (400 nm) plus rPrP (800nM) for 7 days. Human neurons were collected and P-eIF2α (A), P-Erk1/2 (B) and P-PERK (C) protein levels were assed via western Blot. The western blot image quantification of the respective genes is shown below.

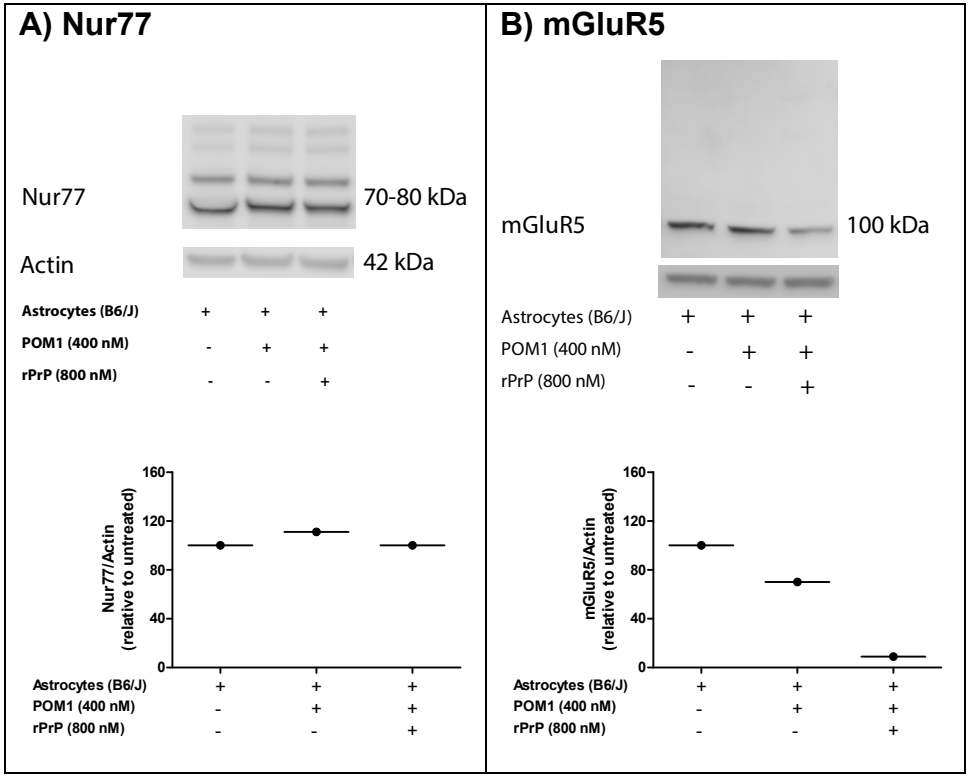


Figure 14: POM1 co-culture human neurons – mouse astrocytes

iPSC derived human neurons were co-cultured with primary mouse astrocytes and treated every other day with POM1 (400 nm) or POM1 (400 nm) plus rPrP (800nM) for 7 days. Human neurons were collected and Nurr77 (A) and mGluR5 (B) protein levels were assed via western Blot. The western blot image quantification of the respective genes is shown below.

5.5.5. Effect of POM1 treatment on astrocytes

In vivo studies suggest that astrocyte specific PrP^C expression is sufficient to induce prion mediated neurodegeneration (Raeber et al., 1997; Jeffrey et al., 2004). One hallmark of prion disease is astrogliosis (Budka, 2003; Liberski and Brown, 2004; Soto and Satani, 2011). LCN2 (lipocalin-2) and GFAP are genes highly upregulated at early stages of astrocytosis (Zamanian et al., 2012). To examine if astrocyte or astrocyte/microglia co-cultures become reactive after POM1 treatment, they were transfected with commercially available plasmids expressing the Firefly luciferase under the GFAP or LCN2 promoter and a control plasmid that expresses the Renilla luciferase under a ubiquitous promoter. One day after transfection, POM1 was added to the astrocytes and astrocyte/microglia co-cultures. Pre-incubation with POM2 or rPrP was used to inhibit the effect of POM1. The expression level of LCN2 and GFAP were determined after an incubation time of 48h and the consecutive addition of the substrates for the Firefly and Renilla luciferase. No significant induction upon POM1 treatment of the astrogliosis markers could be observed in the cultures (see Figure 15). This suggests that astrocytes do not become reactive after POM1 treatment.

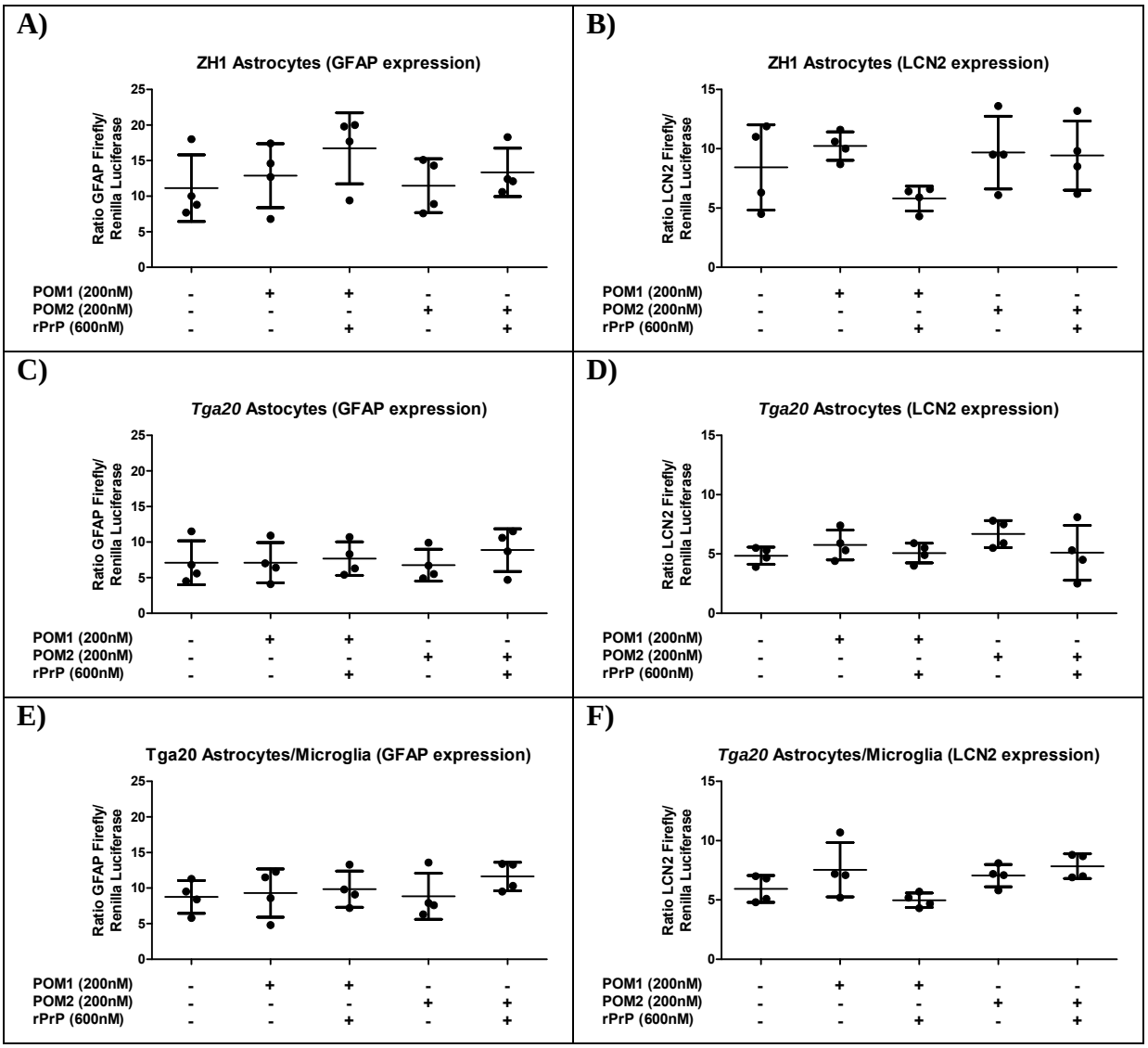


Figure 15: POM1 treatment does not induce astrogliosis

Primary astrocytes that are PrP^C deficient (ZH1) (A-B) or overexpress (tga20) (C-D) or PrP^C overexpressing astrocyte/microglia co-cultures (E, F) were transfected with reporter plasmids that express the Firefly luciferase under the GFAP or LCN2 promoter and a control plasmid that expresses the Renilla luciferase under a ubiquitous promoter. After 1 day the transfected cells were treated with POM1 (200 nM), POM2 (200 nM), POM1 (200 nM) plus rPrP (600 nM), POM1 (200 nM) plus POM2 (200 nM) and POM2 (200 nM) plus rPrP (600 nM). 48h later the LCN2 and GFAP levels were assessed by consecutively adding the substrate for the Firefly and Renilla luciferase. No significant changes could be observed in the expression level of LCN2 and GFAP.

Each error bar indicates average SD of 4 technical replicates. A one way ANOVA with a Bonferroni post-hoc test was used for this experiment (compared groups: Untreated – POM1, Untreated – POM2, POM1 – POM1 + rPrP, POM2 – POM2+ rPrP).

5.6. Discussion

To date prion toxicity has not been observed in a pure neuronal cell culture (Forloni et al., 1993; Muller et al., 1993; Cronier et al., 2004; Cronier et al., 2012) suggesting that a non-cell autonomous mechanism is responsible for neurodegeneration in prion

disease. In my studies I was particularly interested in the involvement of astrocytes in PrP^C mediated toxicity.

POM1, an antibody binding to the globular domain of PrP^C has successfully reproduced prion toxicity in organotypic brain slices (Sonati et al., 2013; Herrmann et al., 2015). Therefore, this antibody was applied to various primary, mESC derived and iPSC derived cell culture systems to explore which subset of cells are necessary for mediating prion toxicity. However, POM1 was unable to trigger neuronal loss under the conditions tested (see Figure 16).

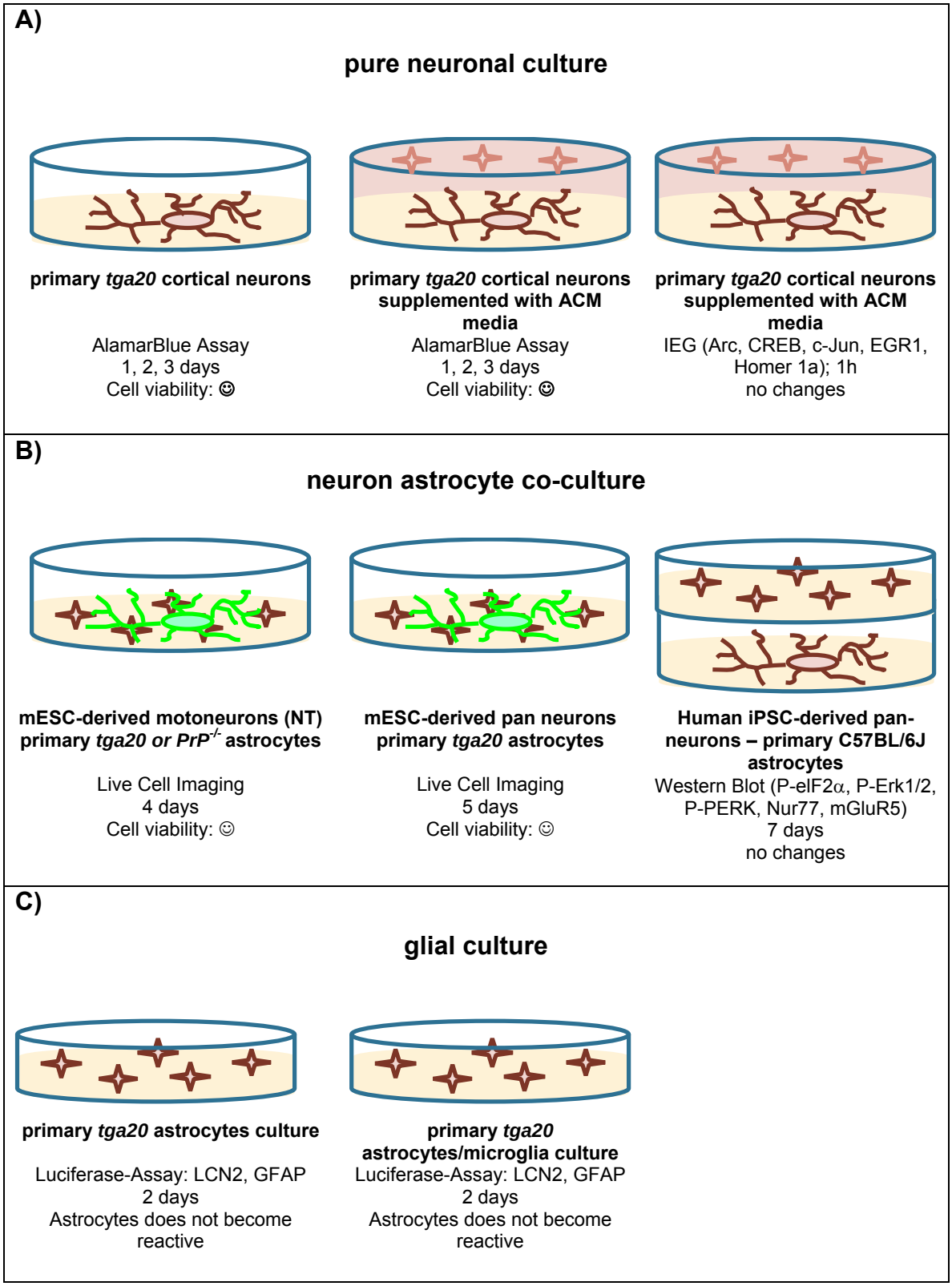


Figure 16: Overview POM1 treatment in vitro

A) – C) POM1 mediated toxicity was tested in mouse primary cortical neuronal cultures supplemented with or without ACM, mESC derived neurons in co-culture with mouse primary astrocyte, hiPSC derived neurons in co-culture with mouse primary astrocytes. D) In addition, the potential of astrocyte to become reactive after POM1 treatment was investigated. However, in none of these conditions changes upon POM1 treatment could be observed.

Table 2: Overview of *in vitro* POM1 toxicity experiments – POM1 toxicity experiments in different neuronal populations
So far we did not observe POM1 mediated toxicity in the different neuronal population listed in table 1.

Neuron type	Species	PrP^C level neurons	Longest treatment time	Cell viability assed via	Molecular changes	POM1 induced toxicity observed
Pure Neuronal Culture						
Cortical neurons	Mouse	Overexpression	3 days	AlamarBlue, LDH Assay	-	No
Astrocyte Conditioned Media						
Cortical neurons	Mouse	Overexpression	3 days	AlamarBlue, LDH Assay	-	No
Cortical neurons	Mouse	Overexpression	1h	-	Arc, CREB, c-Jun, EGR1, Homer1a	No
Neuron-Astrocyte Co-Culture						
Motoneurons	Mouse	WT	4 days	Neuronal morphology	-	No
Pan-Neurons	Mouse	WT	5 days	Neuronal morphology	-	No
Pan-Neurons	Human	WT	7 days	-	P-Erk1/2 P-Perk, p-eIF2α, mGluR5, Nur77	No

Based on the experiments from Sine Yaganoglu and Dr. Tracy O'Connor it was suggested that astrocytes secrete one or more factors that mediate toxicity in cortical neurons. However, they could not reliably reproduce their results with the cytotoxicity assay (LDH-assay) they used. After the evaluation of several cell viability/cytotoxicity assays for this purpose, it was decided to use the alamarBlue reagent. However, I could not detect any toxic effect upon POM1 treatment of cortical neurons in ACM. A possible explanation is that the factor(s) in the ACM are too diluted to trigger toxicity or alternatively, that the assay is not sensitive enough to reveal minor changes in neuronal loss. Furthermore, it is possible that a direct neuron-astrocyte interaction is necessary for POM1 associated cell death. Therefore, it was decided to switch to neuron astrocyte co-cultures to assess POM1 mediated toxicity. However, most of the kits measuring cell viability/cytotoxicity are non-specific to neuronal loss and only detect the viability/cell death in the whole cell population. Thus, we switched to imaging of fluorescent labeled neurons and quantification of cell number and morphological changes for the detection of neurodegeneration. mESC derived moto- and pan-neurons expressing wild type PrP^C did not reveal any changes in cell number or neuronal morphology that indicate neuronal loss after application of POM1.

Another approach to detect POM1 mediated neurodegeneration is the evaluation of transcriptional changes in cortical neurons supplemented with AM or ACM. For this experiment, 1h after POM1 treatment samples were collected and qRT-PCR of the IEG Egr1, Arc, CREB, c-Jun and Homer1a, which are immediate early genes associated with neurodegenerative diseases and/or synaptic plasticity, was performed (Brakeman et al., 1997; Kato et al., 1998; Dickey et al., 2003). However, none of these genes changed in the expression level. This finding could be due to the fact that ACM alone is not sufficient to mediate POM1 dependent neurodegeneration. Furthermore, the expression pattern of the gene might be only changed during a particular time frame.

For the assessment of translational changes upon application of POM1, it was decided to use human neurons co-cultured with mouse astrocytes, expressing wild type PrP^C. Molecular biological studies of genes known to be changed after prion infection and POM1 treatment (P-Perk, P-eIF2α, P-Erk1/2, Nur77) (Herrmann et al., 2015) as well as mGluR5 which mediate neurodegeneration via PrP^C interaction in Alzheimer's disease (Um and Strittmatter, 2013) were not significantly changed.

The absence of POM1 mediated neuronal loss in these experiments could be due to of several reasons. Motoneurons in particular are not very vulnerable to prion infection *in vivo* (Worrall et al., 2000; Budka, 2003). So the absence of toxicity would even confirm that POM1 resembles prion toxicity. However, in that case, at least in mouse and human pan-neuronal cultures with primary astrocytes, we would have expected that a proportion of neurons would have been affected by POM1 toxicity. The reason that we don't observe a loss might be due to the surviving neurons that mask the effect of POM1.

5.7. Outlook

Within the incubation time and concentration of POM1 used, no prion mediated toxicity could be detected in mouse primary, mouse ESC derived or human iPSC derived neurons in co-culture with astrocytes. This is surprising because neuronal loss could be observed in primary astrocyte neuronal co-cultures treated with prion infected brain homogenate (Cronier et al., 2004; Cronier et al., 2012). However it could be that the neurodegeneration was induced via cytokines or other toxic components from the brain homogenate.

In the following section, possible approaches are listed to achieve POM1 mediated toxicity *in vitro* in post-mitotic neuronal cells. One opportunity to achieve POM1 induced neurodegeneration is a mixed brain culture containing various cells resident in the brain. However, for that system it would be necessary to label the neurons or co-culture neurons in a mixed culture to specifically detect neuronal cell death. In case of neuronal cell death in the mixed culture through depletion of specific subtypes of cells (e.g. microglia, astrocytes) it could give an indication of which cell types are important for POM1 mediated toxicity. However, this approach is hampered by the fact that faster proliferating cells like fibroblasts overgrow these cultures and might deplete the effect of the cells important for POM1 mediated neurodegeneration. Morphology, cell signaling pattern, gene expression and polarity could be changed in 2D cultures compared to in vivo system (Edmondson et al., 2014; D'Avanzo et al., 2015). This might be one reason why POM1 mediated toxicity could not be observed in cell culture. An opportunity to reduce these changes is to culture the cells in 3D embedded in matrices generated with the help collagen, Matrigel and self-assembling peptides (SAP) like RADA16 (Liedmann et al., 2012). Especially the use of thin 3D cultures is well suitable for high throughput screenings (Seidel et al., 2012; Kim et al., 2015b).

5.8. Material and Methods

All reagents are purchased at Thermo Fischer if nothing else is stated.

5.8.1. Mice

The housing of mice and animal experiments were performed according to Swiss Animal Protection Law and in compliance with the regulations of the veterinary office (canton Zurich). For our studies the following mice were included *Prnp*$^{ZH1/ZH1}$ mice (Bueler et al., 1992) and *Prnp*$^{o/o}$;*tga20*$^{+/+}$ (*tga20*) (Bueler et al., 1992; Fischer et al., 1996).

5.8.2. Primary cell culture

5.8.2.1. Primary cortical neurons

The primary cortical neurons were prepared according to a protocol from previous Post-Doc, Tracy O'Connor. Pregnant mice with E15.5 - E16.5 neurons were sacrificed and dissected down the midline to remove the uterus. The uterus was placed in Balanced Salt Solution (BSS; 10 mM HEPES, 1% Pen-strep in 1x Hank's

balanced salt solution). After decapitating the embryos, the brain was isolated from skin and skull. Brains were transferred into fresh BSS to separate cortices from the rest of the brain. Meningee-free cortices were digested in Trypsin-solution (0.5 ml 2.5 % Trypsin and 4.5 ml BSS) at 37°C for 15 min and washed three times with BSS. Cortices were triturated using a normal and then a fire-polished Pasteur pipette. Based on the experiment different amounts of cells were seeded into plates pre-coated with 1 mg/mL poly-L-lysine.

Cells were maintained in cortical neuron media (10% horse serum, 1% pen-strep, 1x B-27 supplement (Invitrogen) and 500 μM glutamine in Neurobasal medium (Invitrogen). After four hours, neurons were switched to Neurobasal medium containing 1x B-27 supplement and 500 μM glutamine. Medium was replaced every 2-3 days.

5.8.2.2. Primary astrocytes protocol 1

Primary astrocytes were prepared according to a protocol of Tracy O'Connor. P1-P3 mice were decapitated and brains were isolated in Balanced Salt Solution (BSS; 1x Hank's Balanced Salt solution, 10mM HEPES, 1% P/S). After removing of the meninges, the cortices were digested with 0.5 ml 2.5 % Trypsin in 4.5 ml BSS for 15 min at 37°C. For trituration astrocyte media (10% horse serum, 1% P/S q.s. Opti-MEM) were added and after dissociation the cells were seeded into a 75cm^2 flask (cortices from about 3 brains per flask). The next day the medium was changed and cells were maintained in astrocyte media. ACM used in all experiments in the study was collected from astrocytes prepared according to this protocol.

5.8.2.3. Primary astrocytes protocol 2

The primary astrocytes were prepared according to a modified primary astrocyte protocol 1 (denoted 'Primary astrocytes protocol 2), which was provided by Dr. Vijay Chandrasekar. P1-P3 mice were decapitated and brains were isolated in Balanced Salt Solution (BSS; 1x Hank's Balanced Salt solution, 10mM HEPES, 1% P/S). After removing of the meninges the cortices were minced and put for digesting with 0.25 % Trypsin for 15 min at 37°C. For trituration astrocyte media (10% horse serum, 1% P/S, 1.4% Glucose (45%) q.s. MEM) were added and after dissociation the cells were passed through a 70 um cell strainer. Cortices from about 3 brains were plated into a 75cm^2 flask pre-coated with Poly-D-Lysine (50 ug/ml, ≥ 30 min, 37°C, following three

consecutive washes with PBS). The next day the medium was changed and cells were maintained in astrocyte media.

5.8.3. ACM Collection

To obtain ACM astrocyte media, 10% horse serum, 1% P/S, 1.4% Glucose (45%) q.s. MEM was added to primary astrocyte cultures and after a certain time frame (normally 2 days) the media was collected and sterilely filtered to remove cell debris

5.8.4. Collection of RNA for qRT-PCR

Cortical neurons were grown in a 6-well format (600'000 cells/well). They were exposed to POM1, POM1 plus rPrP and rPrP in ACM at various time points. For RNA collection, samples were lysed in 350 ul/well RLT buffer from the RNeasy Mini-Kit (Qiagen) and frozen in liquid nitrogen. RNA purification was performed using the RNeasy Mini-Kit (Qiagen). Reverse transcription was done with the QuantiTect Reverse Transcription Kit (Qiagen). Used housekeeping gene primers are listed in Table 3 and primers to detect IEG are listed in Table 4.

For qRT-PCR the following conditions were used:

Table 3: Primer house keeping genes for qRT-PCR

Primer name	Gene	Function	Sequence (5'→3')	Amplicon length (bp)	Reference
Ppib fwd	Ppib	secretory pathway	GGAGATGGCACAGGAGGAAA	73	(Kosir et al., 2010)
Ppib rev			CCGTAGTGCTTCAGTTTGAAGT TCT		
Hmbs fwd	Hmbs	Heme synthesis, porphyrin metabolism	TCCCTGAAGGATGTGCCTA	73	(Kosir et al., 2010)
Hmbs rev			AAGGGTTTTCCCGTTTGC		
Hprt1 fwd	Hprt1	Purine synthesis	TCCTCCTCAGACCGCTTTT	90	(Kosir et al., 2010)
Hprt1 rev			CCTGGTTCATCATCGCTAATC		
Eif2a fwd	Eif2a	Protein translation	CAACGTGGCAGCCTTACA	74	(Kosir et al., 2010)
Eif2a rev			TTTCATGTCATAAAGTTGTAGGT TAGG		
Utp6c fwd	Utp6c	Rn18 s biogenesis	TTTCGGTTGAGTTTTTCAGGA	75	(Kosir et al., 2010)
Utp6c rev			CCCTCAGGTTTACCATCTTGC		
B2m fwd	B2m	Histocompatibility	GGTCTTTCTGGTGCTTGTCTCA	103	(Wang et al., 2010a)
B2m rev			GTTCGGCTTCCCATTCTCC		
Gusb fwd	Gusb	Carbohydrate metabolism	CCGATTATCCAGAGCGAGTATG	197	(Wang et al., 2010a)
Gusb rev			CTCAGCGGTGACTGGTTCG		
Tbp fwd	Tbp	protein peptidyl-prolyl isomerization	CAAACCCAGAATTGTTCTCCTT	131	(Wang et al., 2010a)
Tbp rev			ATGTGGTCTTCCTGAATCCCT		
Gapdh fwd	Gapdh	Glicolysis and gluconeogenesis	TCCATGACAACTTTGGCATTG	72	Platform genomics – University of Geneva
Gapdh rev			CAGTCTTCTGGGTGGCAGTGA		

Table 4: primer IEG for qRT-PCR

Primer name	Gene	Sequence (5'→3')	Amplicon length (bp)	Reference
Arc fwd	Arc	TGCAATACAGTGAGGGTACACT	107	fwd: Sine Yaganoglu rwd: (Santini et al., 2011)
Arc rwd		TCCCGCTTGCGCCAGAGGAACT		
CREB fwd	CREB	CTGCCTGAAAGCAACTACAGAAT	111	Sine Yaganoglu
CREB rwd		TCTCTTCATGATTCCTGGCGCT		
Egr1 fwd	Egr1	CGAGTTATCCCAGCCAAACG		Sine Yaganoglu
Egr1 rwd		GAGGCAGAGGAAGACGATGA		
Homer1a fwd	Homer 1a	GAAGTCGCAGGAGAAGATG	134	fwd: (Mahan et al., 2012) rwd: Sine
Homer1a rwd		AGTTCTGTGTCACATCGGGTGT		
c-Jun fwd	c-Jun	CACCAACTGCTTGGATCC		Sine Yaganoglu
c-Jun rwd		AGGAAAGTTGCTGGCCGGTA		

qRT-PCR parameters:
Hold stage: 50 °C, 2 min; 95°C, 10 min
PCR stage (40 cycles): 95 °C, 15 sec; 60°C, 1 min
Melt curve: 95 °C, 15 sec; 60°C, 1 min, 95 °C, 1 min

5.8.5. Preparation of astrocytes for sorting mESCs

At least one day before sorting mESCs, 96-well plates were pre-coated with Poly-D-Lysine (50 ug/ml, ≥ 30 min, 37°C, following three consecutive washes). 10'000 astrocytes were plated per well.

5.8.6. Derivation of neurons from mESCs

In vitro derivation of mESC was performed according to a modified protocol (Wichterle et al., 2002; Ying et al., 2003), provided by Vijay Chandrasekar. Irradiated mouse embryonic fibroblasts (MEFs; CF-1 MEF 4M IRR, GSC-6001G, ams biotechnology) were seeded on Petri dishes pre-coated with 0.1 % gelatin (ES-006-B, Millipore) and supplemented with MEF medium (10 % FBS, 1 % P/S, 1x Glutamax, 5 ml MEM-nonessential amino acids (NEAA) q.s. DMEM). The mESCs were expanded on irradiated MEFs maintained in ESC-media (90 ml ES Cell FBS, 6 ml 2-Mercapthoenthanol, 5 ml P/S, 6 ml NEAA, 5 ml Glutamax, Leukemia Inhibitor Factor (LIF) q.s. KNOCKOUT-DMEM). For generation of EBs the mouse embryonic stem cells were trypsinized and re-suspended in EB-media (50 ml KOSR, 5 ml P/S, 4 ml 2-Mecrapthoenthanol, 3.75 ml Glutamax, q.s. DMEM/F12) on day 1. To promote cellular aggregation into EBs, cells were transferred into bacterial dishes and put into suspension cultures.

Derivation of moto- and interneurons

Derivation of moto- and interneurons was based on a from Vijay Chandrasekar modified protocol (Wichterle et al., 2002). On day 3 and 5 the EB-medium was exchanged and retinoic acid (0.1 mM RA; 1:10 000) and smoothed agonist (0.25 uM SAG; 1:1000, VWR Internat. AG Life Sciences 566660) were added to drive neurons towards a moto-/interneuron linage. At day 7, EB-medium was exchanged and GDNF (0.1 ng/ml) was added. At day 8 the EBs were collected and allowed to settle in a Falcon tube. After careful removal of the media, Papain (Worthington) was added and cells were digested at 37°C for 5 min. Following gentle re-suspension, cells were filtered through a 70 um cell strainer and loaded on an Ovomucoid-Inhibitor gradient (Worthington). After centrifugation at 500 rcf for 5 min media was aspirated and cells were re-suspended in FACS media (2 % horse serum, 1 % P/S q.s. DMEM/F12).

Derivation of pan-neurons

The derivation of the pan-neurons is similar as for the moto- and interneurons. However the induction pattern is different. For the derivation into a pan-neuronal lineage RA and Forskolin were added on day 5 and 7 and on day 9, 11 and 13 the cells were supported with GDNF according to modified protocol from Vijay Chandrasekar (Ying et al., 2003).

mESC derived neurons were maintained in neuron media (50 ml horse serum, 1x N2 supplement, 1x B27 supplement, 1 % P/S, q.s. DMEM/F12). The trophic factors GDNF, BDNF (brain-derived neurotrophic factor), CNTF (ciliary neurotrophic factor) and NT-3 (Neurotrophin-3) were added to a final concentration of 0.1 ng/ml, if not stated otherwise.

5.8.7. FACS-sorting

The cells were sorted with a BD FACS Aria™ III sorter (BD Bioscience) using a 75 or 100 um nozzle. The cells were directly sorted into 96 well plates containing astrocyte. One day after plating, medium was exchanged and, dependent on the experiment, trophic factors were added.

5.8.8. Derivation of iPSCs derived pan-neurons

Derivation of mESC was performed according to a protocol form Dr. Vijay Chandrasekar. Irradiated mouse embryonic fibroblasts (MEFs; CF-1 MEF 4M IRR, GSC-6001G, ams biotechnology) were seeded on Petri dishes pre-coated with 0.1 % gelatin (ES-006-B, Millipore) and supplemented with MEF medium (10 % FBS, 1 % P/S, 1x Glutamax, 5 ml MEM-nonessential amino acids (NEAA) q.s. DMEM). The iPSCs were expanded on irradiated MEFs maintained in iPSC-media (390 ml KNOCKOUT-DMEM, 100 ml knockout serum, 1% Glutamax, 5 ml NEEA, 40 ug fibroblast growth factor recombinant human protein). On day 1 iPSCs were trypsinized and re-suspended in human EB-media (390 ml KNOCKOUT-DMEM, 100 ml knockout serum, 1% Glutamax, 5 ml NEEA). Cells were transferred to low attachment flasks to promote cellular aggregation into EBs. On day 11, 13, 15, 17 and 19 RA (10 nM) was added. From day 15 onwards, cells were additionally supported with GDNF (1 ng/ml). On day 21 EBs were collected and allowed to settle in a Falcon tube. After careful removal of the media, papain (Worthington) was added and cells were digested at 37°C for 1h. Following gentle re-suspension cells were filtered through a 70 um cell strainer and loaded on an Ovomucoid-Inhibitor gradient (Worthington). After centrifugation at 500 rcf for 5 min media was aspirated and cells were re-suspended in human pan-neuronal media (1x N2 supplement, 1x B27 supplement, 4 ml D-glucose 45%solution, 4 ml ascorbic acid (50 mM), 500 ul GDNF (1 ug/ml), 500 ul BDNF (1 ug/ml), 500 ul CNTF (1 ug/ml), 500 ul NT-3 (1 ug/ml) q.s. DMEM/F12). Cells were plated on laminin/lysine pre-coated plates and maintained in human pan-neuronal media.

5.8.9. Preparation of astrocytes for iPSCs derived neurons co-culture

Primary astrocytes were washed once with PBS. After digestion with trypsin 700’000 cells were plated in Poly-D-Lysine pre-coated inserts (Millipore). One day after plating astrocyte media was changed. Three days after seeding, astrocytes they were put in co-culture with 6 week old iPSC derived neurons in human neuron media with B-27 minus antioxidants (1.5 ml per insert and 2 ml per well of a 6 well plate).

5.8.10. Treatment of iPSCs derived neurons with POM1

iPSCs derived neurons in co-culture with astrocytes were treated with 400 nM POM1. Complete media supplemented with fresh POM1 was changed every other day. 7

days after treatment cells were collected for western blot in RIPA-Buffer containing protease and phosphatase inhibitors.

5.8.11. Morphological analysis

Live cell images were processed with the neurite outgrowth program of the Meta Morph Software (Molecular Devices).

5.8.12. Western blot

The cells collected in lysis buffer were homogenized for 30s (frequency 30 Hz) and put for 1 min on ice. This step was repeated 3 times and followed by three consecutive freeze thaw cycles in liquid nitrogen. In order to remove the cell debris the samples were centrifuged at 15'000 g for 15 min at 4°C. For quantification of the protein concentration a BCA-Assay (Pierce) was done according to the manufacture's protocol. 25 ug sample was diluted in loading dye and DTT (final concentration 100 mM) and heated for 5 min at 95°C. After a quick spin the samples were loaded on the gel (Novex, NuPAGE, Bis-Tirs gel) and SDS gel electrophoreses run. The samples were transferred to a polyvinylidene difluoride (PVDF) membrane with the iBlot dry blotting system (life technologies). Membranes were blocked with blocking buffer (5% Top Block diluted into PBS-Tween). After 1h blocking the membranes were incubated with the primary antibody (mGluR5 (Abcam, # ab53090), Nur77 (CST, # 3960S), P-eIF2α (CST, # 9721S), P-Erk1/2 (CST, # 4370S), P-Perk (CST, # 3179S)) diluted in blocking buffer overnight at 4°C. After 3 washing steps with blocking buffer the membrane was incubated with the secondary antibody for 1-2 h followed by 4 consecutive washing steps. The membrane was developed with Western HRP Substrate Luminata Crescendo (Millipore) for 4 min. The blots were imaged with the Stella (Raytest) and semi-quantitative analysis was done with the Quantity-One (Bio-Rad) software.

5.8.13. Preparation of astrocytes or astrocyte/microglia cultures for Dual-Luciferase reporter assay

Primary astrocytes were washed once with PBS and fresh media was added. Astrocyte cultures were placed on an orbital shaker for 3h to remove microglia or mixed astrocyte/microglia cultures were split directly into a 96 well plate (10'000 cells/well). One day after plating, cells were transfected with the LCN2-Firefly

Luciferase (addgene, #25463) and Renilla Luciferase plasmid or with GFAP-Firefly Luciferase (addgene, #40589) and Renilla Luciferase plasmid. For transfection Lipfectamine 3000 was used. The DNA ratio LCN2 or GFAP to Renilla plasmid was 9:1.

5.8.14. AlamarBlue Assay

Experiments were performed in a 96 well plate containing 90 ul of media. 6h before the defined time point 10 ul of alamarBlue was added. Following a 6h incubation, plate fluorescence was assessed with an EnVision Reader (Perkin Elmer) using 560EX nm/590 EM nm filter settings or 531 EX nm/590 EM nm filter settings (plus mirror through which only EX < 555 nm pass and only Em > 555 nm pass through).

5.8.15. Dual-Luciferase reporter assay

The Dual-Luciferase reporter assay (Promega) was used for quantification of Luciferase activity. For lysis, astrocyte media was removed and 25 ul Passive Lysis Buffer (PLB) was added. After 15 min shaking (1000 rpm, 20 °C) Luciferase Assay Reagent (LAR) containing the Luciferase substrate was added and 120 ul/well of suspension mix was transferred to a fresh white plate suitable for measuring luminescence. Luciferase activity was measured with an EnVision Reader (Perkin Elmer). The Stop & Glo Reagent, which simultaneously quenches the Firefly Luciferase signal and initiates the Renilla Luciferase reaction, was added and the activity of Renilla Luciferases was measured with the EnVision Reader (Perkin Elmer) according to the manufacture's protocol.

6. Potential function of PrP^C as a cell death transducer

6.1. Proposed physiological role of PrP^C

PrP^C expression starts during early embryogenesis (Manson, 1992; Harris 1993). In adult mammalian species expression is found in many cell types with a particularly high enrichment in neurons (Kretzschmar et al., 1986; Moudjou et al., 2001). Evidence in favor of an important biological function of PrP^C are, the high structural conservation of PrP^C (Roucou et al., 2004), no naturally occurring *Prnp*-null alleles are observed throughout the mammalian kingdom (Aguzzi and Calella, 2009; Benestad et al., 2012). The use of PrP^C knock-out mice has not shed much light on the role of this gene (Weissmann and Flechsig, 2003; Steele et al., 2007). In the Zurich II, Nagasaki and Rcm0 PrP^C knock out mouse strains, cerebellar degeneration and ataxia has been observed that could be rescued by the re-introduction of PrP^C (Weissmann and Flechsig, 2003). While this observation endorses the essential role of PrP^C in maintaining a healthy neuronal status, the Zurich I and the Edinburgh knock out lines fail to demonstrate this striking phenotype (Weissmann and Flechsig, 2003; Linden et al., 2008). In fact, it turns out that, this phenotype represents a genetic artifact introduced by the different strategies used to generate these mouse strains (Linden et al., 2008). In the Zurich I and the Edinburgh lines, ablation of the prion protein has been achieved by modifying the exon 3, which contains the ORF of *Prnp* (Weissmann and Aguzzi, 1999; Weissmann and Flechsig, 2003). A more aggressive approach has been used in the Zurich 2, Nagasaki and Rcm0 PrP^C knock out mouse lines, where the flanking regions of the ORF were also disrupted (Weissmann and Aguzzi, 1999; Weissmann and Flechsig, 2003). Therefore, the acceptor splice site in the *Prnp* gene was lost, which in turn lead to Doppel (*Dpl*) overexpression, encoded by *Prnd* located 16 kDa downstream of *Prnp* (Moore et al., 1999). Dpl is a GPI anchored membrane protein that shares about 25 % sequence similarity with PrP^C (Moore et al., 1999). Interestingly Dpl and PrP^C have common features in the globular domain while an equivalent to the octapeptide region of PrP^C is missing in Dpl (Moore et al., 1999). In wild type mice Dpl is highly expressed in the testis, to some extent in peripheral organs and at very low levels in the brain (Silverman et al., 2000) (see Figure 17).

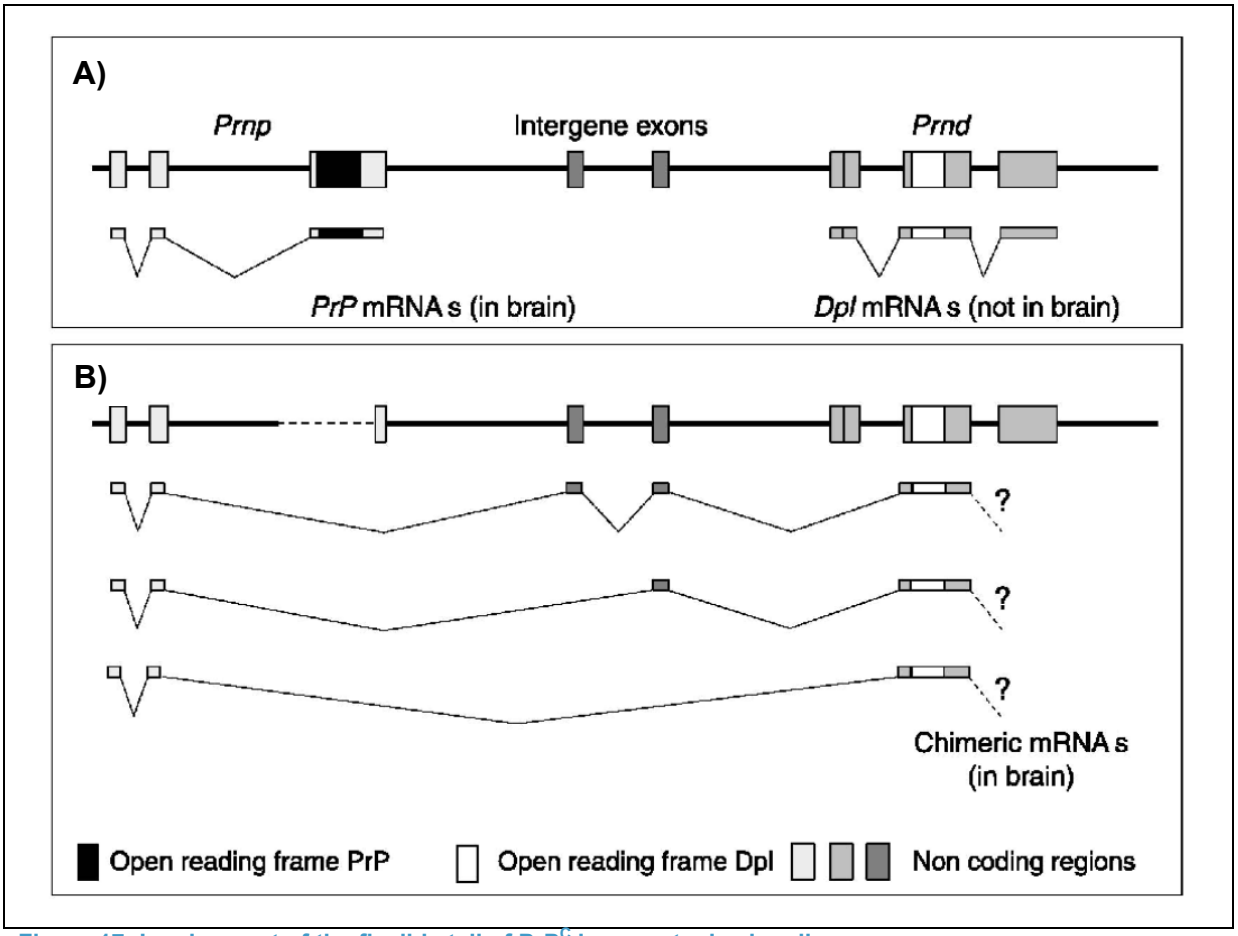


Figure 17: Involvement of the flexible tail of PrP^C in neurotoxic signaling
(A) *Prnd* is encoded downstream of *Prnp* separated by an intergenic region (Weissmann and Flechsig, 2003). (B) Through aggressive depletion of the *Prnp* coding region in exon 3 the splice acceptor site can be lost (Weissmann and Flechsig, 2003). This leads to exon skipping and transcription of chimeric mRNAs encoding *Prnd* regulated by the *Prnp* promotor (Weissmann and Flechsig, 2003),
Figure modified from (Weissmann and Flechsig, 2003)

Besides the phenotypes caused by genetic artifact, the most striking feature common for PrP^C knock out mice is that they are resistant to prion infection (Weissmann and Flechsig, 2003; Steele et al., 2007) and lack a relevant pathological phenotype (Bueler et al., 1993; Prusiner et al., 1993; Sailer et al., 1994). Hypothetical mechanisms underlying this observations include compensation by another protein or that the function of PrP^C is dependent on undefined selective pressures, which would make it challenging to reveal the role of PrP^C (Roucou and LeBlanc, 2005; Steele et al., 2007). Nevertheless, intensive studies of histopathological changes have revealed that depletion of PrP^C causes a chronic demyelinating polyneuropathy (CDP) (Nishida et al., 1999; Baumann et al., 2007; Bremer et al., 2010). More specifically, it has been reported that the neuronal expression and proteolytic

cleavage of the N-terminal region of PrP^C is involved in myelin maintenance (Bremer et al., 2010); (Küffer et al. submitted).

Apart from the generation of PrP^C knock out mice, several other strategies including protein-protein interaction assays and genetic screenings have been used to unmask the physiological function of PrP^C (Steele et al., 2007). However, most of the suggested functions remains controversial and the mechanisms behind the specific interactions are poorly understood (Steele et al., 2007) (see Table 5).

An example is the suggested anti-oxidative function of PrP^C (Steele et al., 2007). The OR region of *Prnp* is able to bind copper (Brown et al., 1997), and based on this observation, many studies have suggested a protective effect of PrP^C against oxidative species (Roucou and LeBlanc, 2005; Steele et al., 2007). However, genetic ablation of PrP^C *in vivo* shows no significant impairment of SOD activity and therefore contradicts this theory (Hutter et al., 2003).

Another hypothesis is that PrP^C regulates the circadian rhythm (Tobler et al., 1996). An altered circadian rhythm and sleeping pattern has been detected in PrP^C knock out mice (Tobler et al., 1996; Huber et al., 1999). Dysregulation of the sleep-wake cycle and sleep reduction are characteristic features of fatal familial insomnia (FFI), an inherited form of prion disease (Gambetti et al., 1995; Montagna et al., 1995; Lugaresi et al., 1998; Montagna et al., 2003). Furthermore, sleep-wake disturbances similar to FFI have also been observed in CJD (Landolt et al., 2006). Other suggested functions include stem-cell renewal and proliferation of neuronal progenitors, neuronal excitability, cell signaling and neurogenesis (Steele et al., 2007) (see Table 5).

In summary, the most likely function of PrP^C is neuroprotection, supporting the hypothesis that loss of function leads to cell death in prion disease.

Table 5: Examples of proposed functions of PrP based on PrP knock out mice

Table modified from (Steele et al., 2007)

Phenotype in PrP knock out mice	Reference	Confirmed?	Opposing?
Myelin maintenance			
chronic demyelinating polyneuropathy	(Bremer et al., 2010)	(Küffer et al., submitted); (Nuvolone et al., 2016)	
Circadian/Sleep regulation			
Altered circadian rhythm	(Tobler et al., 1996)		
Disturbance of sleeping pattern	(Tobler et al., 1997)		
Neuronal excitability			
Altered long term potentiation	(Collinge et al., 1994)	(Carleton et al., 2001; Herms et al., 2001; Curtis et al., 2003; Asante et al., 2004; Criado et al., 2005)	(Herms et al., 1995; Lledo et al., 1996; Maglio et al., 2004; Maglio et al., 2006)
Enhanced susceptibility to seizures	(Walz et al., 1999)	(Rangel et al., 2007)	(Striebel et al., 2013a; Striebel et al., 2013b)
Neuroprotection			
Increased susceptibility to ischemia	(McLennan et al., 2004)		
Increased susceptibility to apoptosis caused by Ethanol	(Gains et al., 2006)		
Increased susceptibility	(Hoshino et al., 2003)		
Stem and precursor cells			
Impaired self-renewal of stem cells	(Zhang et al., 2006)		
Decreased proliferation of neuronal progenitors	(Steele et al., 2006)		

6.2. PrP^C neurotoxicity is mediated by the flexible tail

The flexible tail of PrP^C seems to play an essential role in prion pathogenesis. Prion or POM1 (an antibody against the globular domain of PrP^C) mediated toxicity in organotypic cerebellar slices can be prevented by the application of an antibody targeting the OR within the flexible tail of PrP (POM2 antibody) (Sonati et al., 2013; Herrmann et al., 2015) (see Figure 19 A). This leads to the hypothesis that PrP^C triggers cell death signaling via its flexible tail (see Figure 19 C). Moreover, depletion of this specific region ameliorates the pathology in prion infections (Flechsig et al., 2000) (see Figure 19) and POM1 toxicity (Sonati et al., 2013). The octapeptide repeat insertion in the *PRNP* gene is responsible for inherited prion diseases such as GSS and CJD (Goldfarb et al., 1991; Mead et al., 2006; Kumar et al., 2011). Consistent with these findings is the observation that spontaneous neurodegeneration occurs in mice expressing the amino-terminal truncated PrP^C (Shmerling et al., 1998) or a GPI-linked flexible tail (Dametto et al., 2015). Additionally, it has been found the OR of PrP^C is the binding site for amyloid beta in

Alzheimer's disease (Lauren et al., 2009; Zou et al., 2011). Several investigations indicate that PrP^C is responsible for neuronal loss and memory deficits in Alzheimer's disease (Larson et al., 2012; Um et al., 2012; Ostapchenko et al., 2013; Um and Strittmatter, 2013). Therefore, we decided to query whether PrP^C could act as a general cell death transducer in neurodegenerative diseases (see Figure 19 D). As mentioned before, the N-terminal region of PrP^C is essential for its neurotoxic function (Shmerling et al., 1998; Sonati et al., 2013; Dametto et al., 2015; Herrmann et al., 2015) .Accordingly, if PrP^C acts as a cell death transducer, blocking this specific region could have a beneficial effect on neuronal viability, not only in prion infection and POM1 toxicity, but also against a general plethora of cell stressing agents that cause ER stress and neurotoxicity

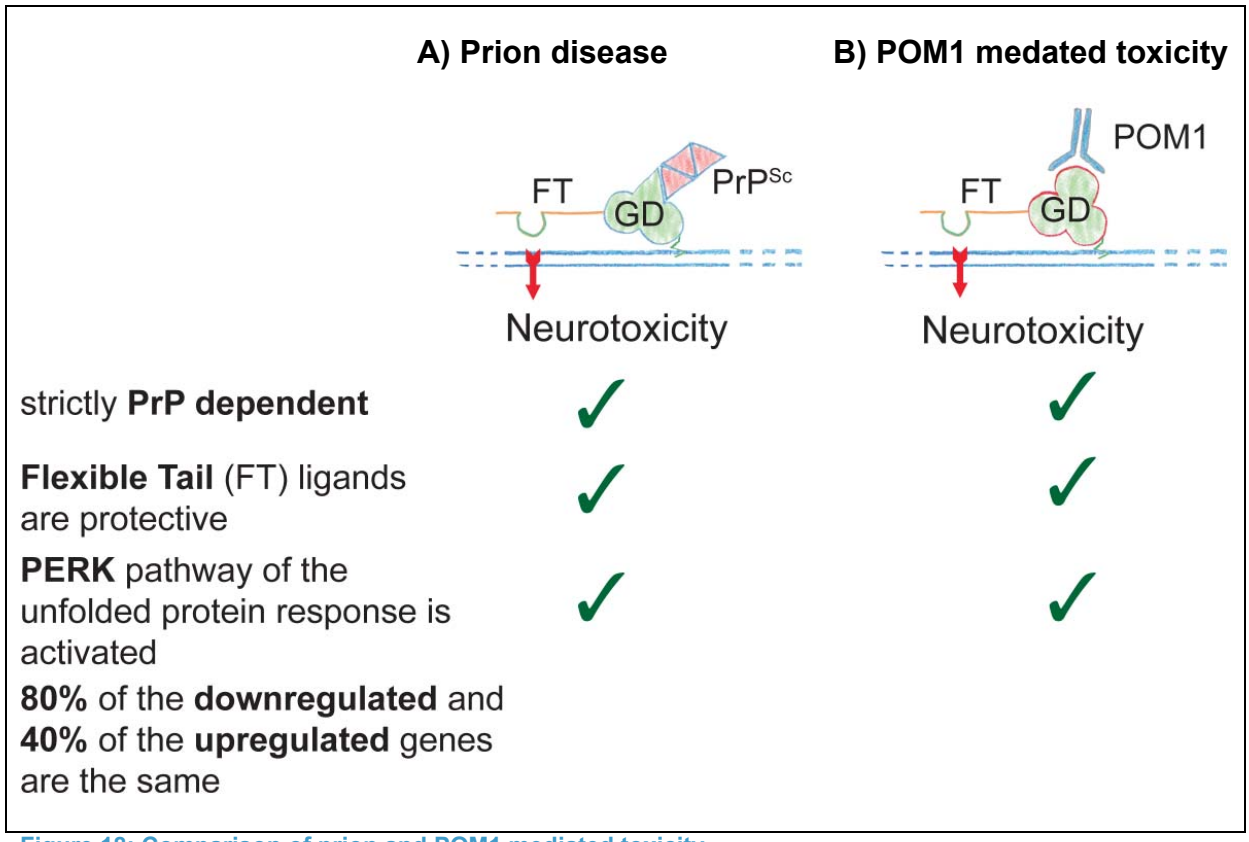


Figure 18: Comparison of prion and POM1 mediated toxicity
Prion disease (A) as well as in POM1 mediated toxicity (B) are both PrP^C dependent, activate the PERK signaling branch of the UPR and have huge set of overlapping genes that are differently upregulated (Herrmann et al., 2015). This suggest that POM1 is a good mimic of prion mediated toxicity (Herrmann et al., 2015).
Image modified: (Herrmann et al., 2015)

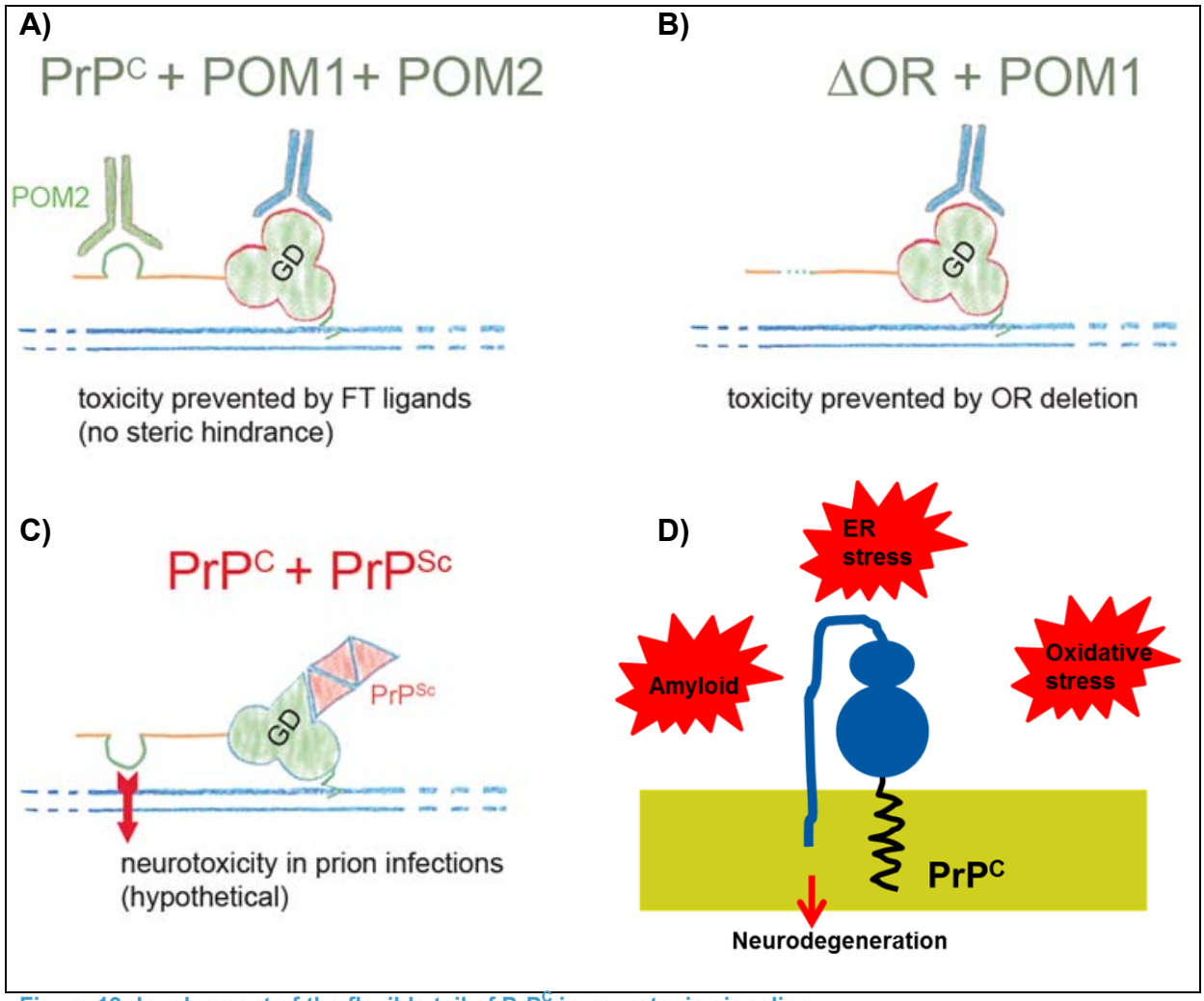


Figure 19: Involvement of the flexible tail of PrP^C in neurotoxic signaling
Pre-incubation of POM2 A) that binds to the octapeptide region (OR) of PrP^C or B) depletion of the OR region blocks POM1 mediated toxicity (Sonati et al., 2013). C) Therefore, Sonati et al. postulated that the flexible tail might be essential for the initiation of neurodegeneration in prion disease (Sonati et al., 2013). D) Based on this observation, it was hypothesized that PrP^C could act as a general cell death transducer initiating cell death upon different cellular stressors like oxidative stress, ER stress and amyloid beta (Illustrations A-C modified (Sonati et al., 2013)).

6.3. ER homeostasis in neurodegenerative diseases

In prion disease as well as in the POM1 mediated toxicity an upregulation of genes involved in the UPR (Unfolded Protein Response) are induced ((Sonati et al., 2013; Herrmann et al., 2015).

6.3.1. Unfolded Protein Response is activated in response to ER stress

ER stress can be triggered by a variety physiological and pathophysiological conditions that lead to the accumulation of misfolded proteins in the ER (Bravo et al., 2013; Sano and Reed, 2013; Hetz and Mollereau, 2014). To counteract ER imbalance, the cell activates a mechanism called the UPR (Rao et al., 2004; Hetz and Mollereau, 2014). The three core signaling proteins of the UPR are inositol-requiring enzyme 1 (IRE1), activating transcription factor 6 (ATF6) and protein kinase RNA-like ER kinase (PERK) (Hetz, 2012; Sano and Reed, 2013; Hetz and Mollereau, 2014). All of these three ER membrane proteins bind to the Immunoglobulin Binding Protein (BIP (GPR78)), which is released into the cytoplasm during the UPR, where it assists in protein folding and thereby activates these three signaling branches (Hetz, 2012). Initially ER stress leads to a translation stop, whereas specific proteins assisting in protein folding and ER-assisted degradation (ERAD) mediated protein decay are upregulated (Kim et al., 2008). If these mechanisms are not sufficient to restore ER homeostasis, cell death is initiated (Kim et al., 2008).

The induction of IRE1 enables unconventional splicing of XBP1 mRNA (Yoshida et al., 2001; Uemura et al., 2009). The spliced from of the transcription factor XBP1 activates genes involved in ERAD mediated decay, protein folding, lipid synthesis and other important processes of the UPR (Hetz et al., 2011). Upon ER stress, ATF6 translocates to the Golgi, where it is activated (Hetz, 2012). The active fragment of ATF6 causes upregulation of genes encoding ERAD components and XBP1 (Hetz, 2012). The autophosphorylation of PERK initiates the phosphorylation of eIF2α, which induces a general translational stop and upregulation of specific mRNA like ATF4 that results in cell death if ER stress persists (Hetz and Mollereau, 2014; Wang and Kaufman, 2016) (see Figure 20).

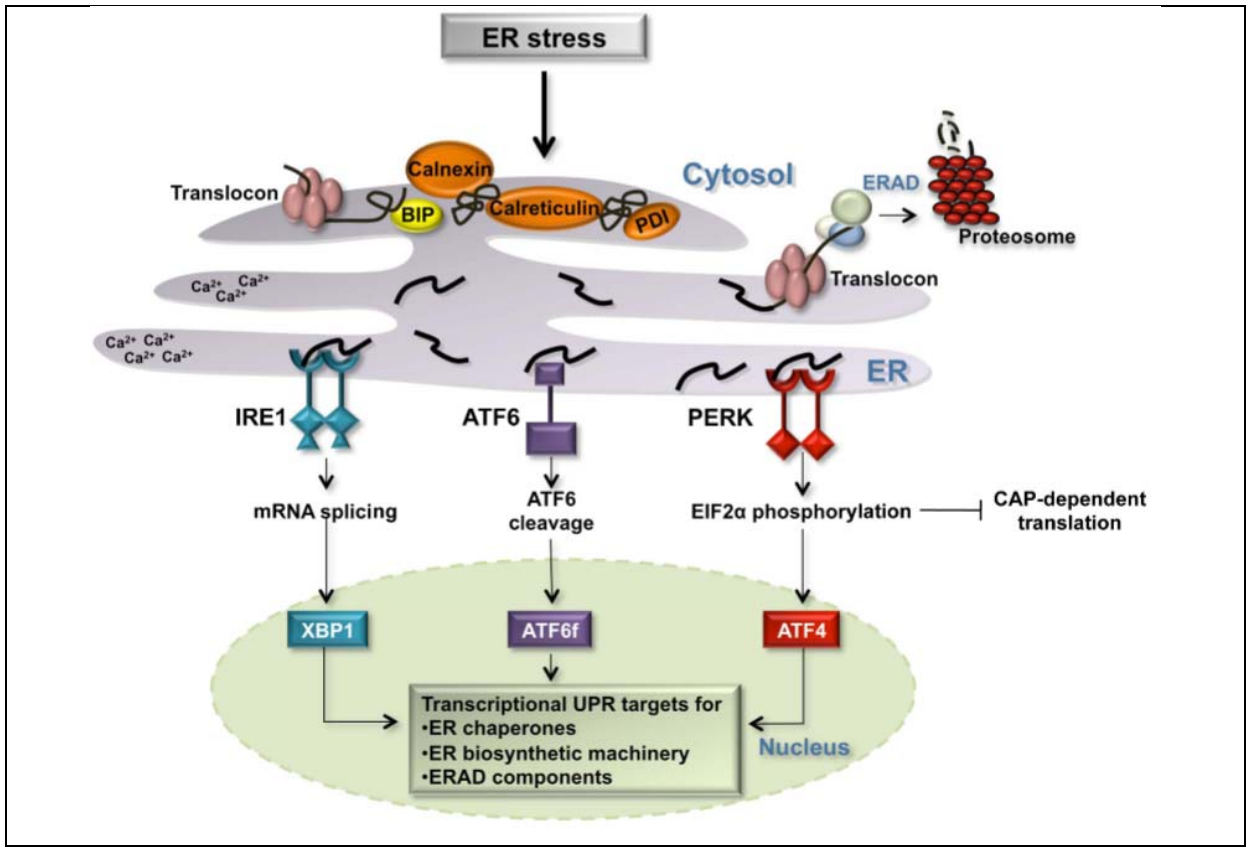


Figure 20: Activation of UPR via three main signaling pathways
ER stress initiates the UPR via three main signaling branches activated by the transmembrane receptors IRE1, ATF6 and PERK (Bernales et al., 2012). The UPR tries to restore ER homeostasis via a global inhibition of translation and activation of translation of genes important for protein folding and ERAD degeneration (Bernales et al., 2012) (Illustration (Bernales et al., 2012))

6.3.2. Activation of ER stress in neurodegenerative diseases

Despite the distinctive clinical and pathological features , neurodegenerative diseases such as Alzheimer disease, Huntington disease, amyotrophic lateral sclerosis (ALS) and prion disease share a common feature: the accumulation of abnormally folded proteins (Soto, 2003; Doyle et al., 2011). Therefore, these diseases are collectively referred to protein misfolding disorders (PMDs) (Chiti and Dobson, 2006).

There is growing evidence that the neuronal losses in PMDs are caused by the disturbance of ER homeostasis, in particular, by malfunctions in the UPR (Hetz and Mollereau, 2014). Therefore, targeting the UPR may offer a new therapeutic option in PMDs (Hetz et al., 2013; Torres et al., 2015). However, caution is merited, since experimental data indicate that different proteins involved in the UPR can have either protective or disease promoting function dependent on the PMD (Hetz et al., 2013; Hetz and Mollereau, 2014; Scheper and Hoozemans, 2015) (compare Table 6).

Table 6: Signaling pathways affected in PMD

Signaling pathway	PMD	UPR marker	Model system	Effect	Reference
IRE1 pathway	Prion disease	XBP1	mouse	ablation reveals no change in the disease phenotype	(Hetz et al., 2008)
	Alzheimer's disease	IRE1	cell lines	PS1 mutants compromise IRE1 signaling	(Katayama et al., 2001)
		XBP1	human	downregulated	(Reinhardt et al., 2014)
	ALS	XBP1	mouse	ablation attenuates disease phenotype	(Hetz et al., 2009)
	Huntington's disease	XBP1	mouse	ablation attenuates disease phenotype	(Vidal et al., 2012)
PERK pathway	Prion disease	PERK eIF2α	mouse	phosphorylated forms of PERK and eIF2α only detected in CJD inoculated mice with neurofibrillary pathology	(Unterberger et al., 2006)
		eIF2α ATF4	mouse brain slices	Upregulation of P-eIF2α and ATF4	(Herrmann et al., 2015)
		GADD34	mouse	Decreased levels of GADD34 lead to continuous activation of eIF2α	(Moreno et al., 2012)
	Alzheimer's disease	PERK	cell lines and primary cells	PS1 mutant cause malfunction	(Katayama et al., 2001)
		GADD34	mouse	upregulation	(Honjo et al., 2015)
	ALS	PERK	mouse	Expression of a non-functional form of PERK accelerates disease progression	(Wang et al., 2011)
		ATF4	mouse	ATF4 promotes disease progression	(Matus et al., 2013)
		GADD34	mouse	Prevention of upregulation of GADD34 is neuroprotective	(Jaronen et al., 2013; Wang et al., 2014)
	Huntington's disease	ATF4	mouse	ablation of ATF4 shows no difference in disease phenotype	(Vidal et al., 2012)
ATF6 pathway	Alzheimer's disease	ATF6	cell lines and primary cells	PS1 mutations prevent activation of ATF6	(Katayama et al., 2001)
	ALS	ATF6	cell lines	VAPB prevents processing of ATF6	(Gkogkas et al., 2008)
		ATF6	human	upregulation of ATF6 levels in spinal cord	(Atkin et al., 2008)
	Huntington's disease	ATF6	mouse	inactivation of ATF6	(Naranjo et al., 2016)

6.3.3. Therapeutic targeting of ER pathways in neurodegenerative diseases

Attenuation of ER stress levels with pharmacological or gene therapy has been successful in reducing the pathological features in various animal models of neurodegeneration and thus holds promise as a therapeutic target for human neurodegenerative diseases (Kim et al., 2008; Hetz and Mollereau, 2014).

For example, the PERK inhibitor GSK2606414 has been shown to ameliorate neuropathological and clinical signs of prion disease (Moreno et al., 2013). Despite the favorable bioavailability of this drug and observed therapeutic potential, GSK2606414 is far from being implemented as a treatment option because of severe side effects such as weight loss and mild hyperglycemia (Moreno et al., 2013). Another tested inhibitor is Salubrinal, which targets the UPR by inhibition of the inactivation of eIF2α (Boyce et al., 2008). While the agent exhibits a protective effect in ALS (Saxena et al., 2009) and Parkinson's disease (Colla et al., 2012), it seems to aggravate the disease phenotype in prion diseases (Moreno et al., 2012).

Apart from small-molecule inhibitors, gene therapies have been tested as promising treatment in PMDs. In a mouse model of Huntington's disease, overexpression of XBP1 leads to a decrease in mutant Huntingtin (Zuleta et al., 2012). Additionally, overexpression of GADD34 leads to a significant extension of the survival time in prion disease (Moreno et al., 2012). An overview of the aforementioned therapeutic interventions is provided in the table below.

Table 7: Therapeutic targeting of the UPR in PMD

Disease	Model	Agent	Effect	Reference
Pharmacological approaches				
Prion disease	mouse	PERK inhibitor	Decreased neurodegeneration, delayed disease progression	(Moreno et al., 2012)
Prion diseases	mouse	Salubrinal	Disease exacerbation	(Moreno et al., 2013).
ALS	mouse	Salubrinal	Extended lifespan	(Saxena et al., 2009)
Gene therapy				
Huntington's disease	mouse	AAV-XBP1s	Reduction of mutant Huntingtin	(Zuleta et al., 2012)
Prion disease	mouse	LV-GADD34	Decreased neurodegeneration	(Moreno et al., 2012)

6.4. Scientific Aims

PrP^C is mainly distinguished by its essential role in mediating neurodegeneration in prion disease (Bueler et al., 1993; Sailer et al., 1994). However, the molecular mechanisms that underlie the neuronal cell loss are still enigmatic (Aguzzi and Calella, 2009). Understanding the physiological function of prion protein may help unravel the pathophysiology of prion disease, which in turn may facilitate the design of effective therapeutic strategies.

The aim of the project was to investigate the involvement of PrP^C in neurotoxic signaling caused by ER stress. Our experimental setup included the following studies:

- ***Study of the protective role of POM2, a N-terminal targeting anti-PrP antibody against ER stress***. POM2 binds to the OR of the PrP^C and is neuroprotective against POM1 and RML induced toxicity in cerebellar slices (Sonati et al., 2013; Herrmann et al., 2015). In addition, it has been suggested that the neuronal loss in prion disease is evoked by disturbed ER homeostasis (Xu and Zhu, 2012; Roussel et al., 2013). Therefore, we sought to evaluate the potential of this antibody to alleviate neuronal loss triggered by ER stress. Dependent on the PMD, different types of neurons have different levels of vulnerability and response to cell stress (Saxena and Caroni, 2011; Jackson, 2014). Therefore, we utilized different types of neurons including moto-, inter- and granule neurons and pre-incubated with POM2 before stressor treatment. The extent of neuroprotection was measured by the morphological assessment of neurite outgrowth and the assessment of neuronal number with significant outgrowth.
- ***Study of the potential of POM2 as a therapeutic target in animal models of neurodegenerative diseases.*** My preliminary data indicate that POM2 delays ER stress *in vitro* and therefore might qualify as a useful therapeutic target of PMD. *However,* there were some indications that POM2 causes hemolysis ((Sonati et al., 2013) and unpublished data of Prof. Magda Polymenidou), an observation that would prohibit the application of POM2 via intravenous injection. In my experiments, I could confirm the aforementioned observations. As a result, it was decided to use an adeno-associated virus (AAV) expressing scFv POM2 for the therapeutic intervention in *in vivo* studies and the viruses were prepared in collaboration with Prof. Bernard Schneider.

- ***Evaluation of the protective potential of POM2 in vivo.*** Testing the therapeutic potential of the AAV expressing scFv POM2 in multiple animal models of neurodegenerative diseases. Since these experiments are tedious and time consuming and result in a severe phenotype (for example, Huntington's or ALS disease models), it was decided to test the therapeutic potential of POM2 first in a rather simple *in vivo* system. Thus, the AAV expressing scFv POM2 was injected to the mouse eye prior to photo toxicity-induced retinal neurodegeneration (collaboration Prof. Christian Grimm).

6.5. Results

6.5.1. In vitro model systems to explore the potential of PrP^C as a general cell death transducer

ER stress is a common feature of neurodegenerative diseases (Lindholm et al., 2006; Doyle et al., 2011; Roussel et al., 2013). PrP^C is involved in neurotoxic signaling in both prion (Bueler et al., 1993; Sailer et al., 1994) and Alzheimer disease (Kudo et al., 2013; Um and Strittmatter, 2013; Jarosz-Griffiths et al., 2016). We sought to test whether PrP^C can act as a general cell death mediator. In order to test the latter, we utilized the protective POM2 antibody (Sonati et al., 2013; Herrmann et al., 2015) in mouse primary or ESC-derived neurons. In neurodegenerative diseases a distinct subset of neurons are usually affected, which defines the clinical symptoms (Saxena and Caroni, 2011; Jackson, 2014). Therefore, several neuronal subpopulations were used to check the ability of POM2 to counteract ER stress. Moto- and interneurons that are vulnerable to neuronal loss in ALS (Saberi et al., 2015) and cerebellar granule neurons, which represent a major type of neuronal population affected in prion disease (Budka, 2003; Faucheux et al., 2009) were used (see Table 8).

The ER stressor Tunicamycin (TM) was selected to assess the protective efficiency of the POM2 antibody. TM is a composite of different homologous nucleoside antibiotics (Price and Tsvetanova, 2007). It specifically inhibits N-acetylglucosamine transferases, which prevents the glycosylation of newly synthesized glycoproteins (Takatsuki et al., 1975; Tkacz and Lampen, 1975). This in turn disables the transport of those proteins from the ER to other cellular compartments, which results in local accumulation or ER stress (Oslowski and Urano, 2011).

Table 8: Types of neurons used for screening the protective effect of POM2

Type of neuron	Species	Reflecting population affected in	Fluorescent marker	Image type used for analysis	Comment
ESC-derived moto- and interneurons	mouse	ALS	GFP expression under HB9 promoter	fluorescent images	Derived from NT mESCs
Primary granule neurons	mouse	Prion disease	No	bright field images	From *tga20* or ZH3 pups

Neurons were treated with different concentrations of TM selected according to previous publications (Galehdar et al., 2010; Nunziante et al., 2011; Dery et al., 2013; Kim et al., 2015a).

For evaluation of neuroprotection, live cell imaging was performed with the Plate Runner (Trophos) or the IncuCyte (EssenBio). Compared to the Plate Runner (Trophos), which was described under 5.5.3.1, the IncuCyte has the advantage that reading can be done directly in the incubator, which avoids temperature fluctuations. Furthermore, up to six 96 well plates fit in the IncuCyte and the reading could be programmed accordingly. The moto- and interneurons expressed a fluorescent marker whereas the granule neurons were not fluorescently labeled. Therefore, for quantification of the moto- and interneurons, fluorescent live-images were used, whereas for the the data analysis of granule neurons were based on bright field images.

Image analysis was performed with MetaMorph software. For quantification of the data, I focused on the cell number with significant outgrowth and changes in the neurite outgrowth, because these parameters exhibited the most significant changes upon stressor treatment (data not shown).

6.5.1.1. Protective effect of POM2 against ER stress

mESC derived moto- and interneurons, expressing wildtype PrP^C, were co-cultured with primary *tga20* astrocytes overexpressing PrP^C. One day after plating the neurons, neuron-astrocyte co-cultures were incubated for 2 h with POM2 prior to ER stress induction. TM (1 ug/ml) treatment resulted in neuronal loss and a reduced neurite outgrowth in moto- and interneurons. This preliminary test showed a more profound stress effect of TM treatment on interneurons compared to motoneurons, as defined by increased neuronal loss and reduced neurite outgrowth. The application of

POM2 to interneurons did not reveal any protective effect against ER stress (see Figure 21 A and B). 12h after stressor treatment, we could observe a significant increase in the number of motoneurons with significant outgrowth, however, only in motoneurons pre-incubated with POM2 (see Figure 21).

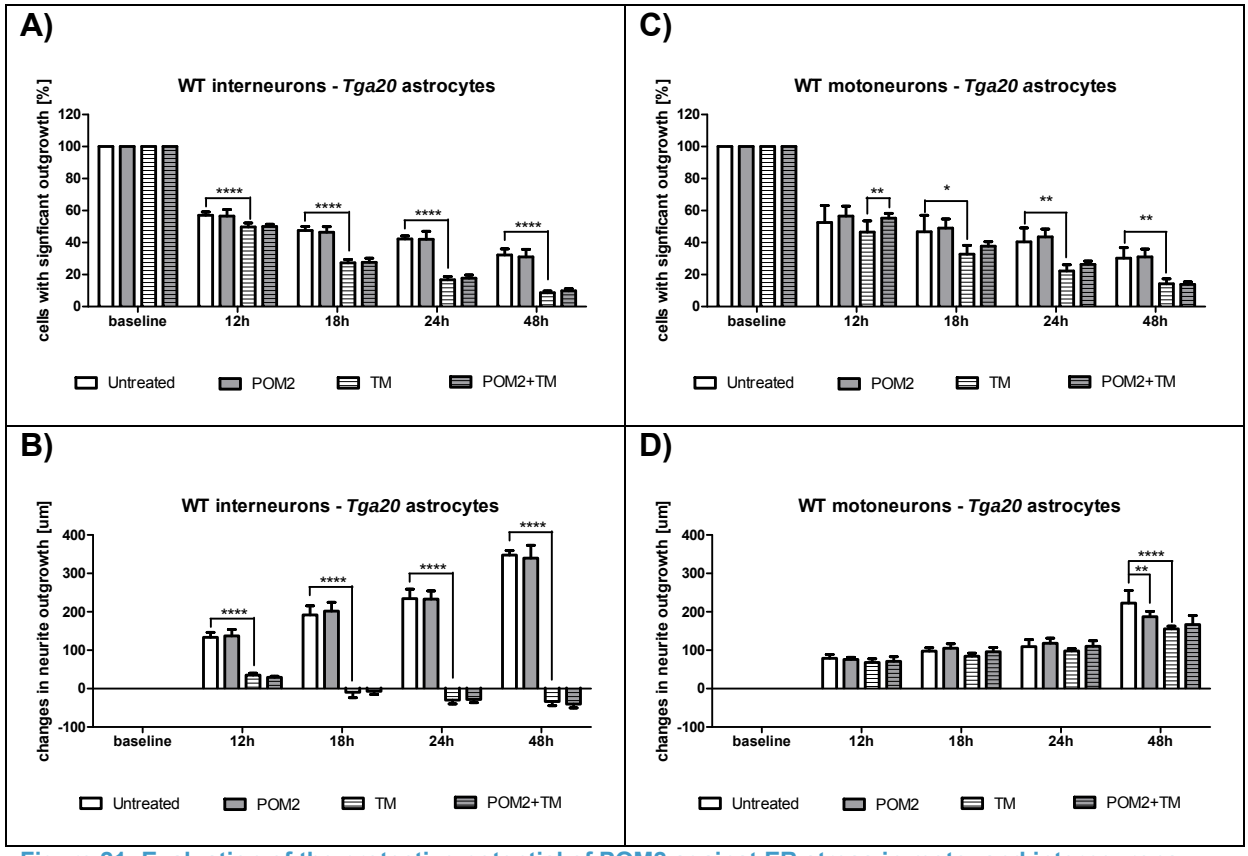


Figure 21: Evaluation of the protective potential of POM2 against ER stress in moto- and interneurons
mESC derived moto- and interneurons were pre-incubated with POM2 (200 nM) for 2h prior to TM treatment (1 ug/ml). To evaluate cell viability, the cell number with significant outgrowth and the changes in the neurite outgrowth were analyzed in co-cultures of astrocytes with interneurons (A and B) or (C and D) motorneurons, respectively. Although TM induced significant changes in neuronal morphology in the interneuron-astrocytic co-cultures (A and B), no protective effect of POM2 could be observed. In the motorneuron-astrocytic co-cultures (C and D), the morphological changes are less significant. 12h after stressor treatment, a significant increase in the number of motoneurons with significant outgrowth was observed, however, only in motoneurons pre-incubated with POM2. *Each error bar indicates average SD of 4-5 technical replicates. The two-way ANOVA with Bonferroni post-test was used for this experiment (compared groups: Untreated-POM2, untreated-TM, TM-POM2+TM) ($p < 0.0001$ = ****, $p < 0.001$ = ***, $p < 0.01$ = **, $p < 0.1$ = *)*

The next step was to elucidate the protective potential of POM2 in pure granule neurons. In contrast to the previous studies where neuron astrocyte co-cultures were used, the use of pure neuronal cultures excludes any non-cell autonomous effect due to glial cells. In these granule neuronal cultures, POM2 was shown to have a positive effect on TM treated cells, although a complete rescue from the toxic effect of the

stressor could not be observed. It appears that POM2 exerts its protection by delaying the induction of ER stress and neurodegeneration.

Compared to motoneurons in co-culture with astrocytes, the protective potential is much more significant, which might be also due to the fact that neurons overexpress PrP^C. In addition, increased neurite degeneration upon TM treatment could be seen, which is most likely accounted for by the 10 times higher TM concentration and the absence of astrocytes.

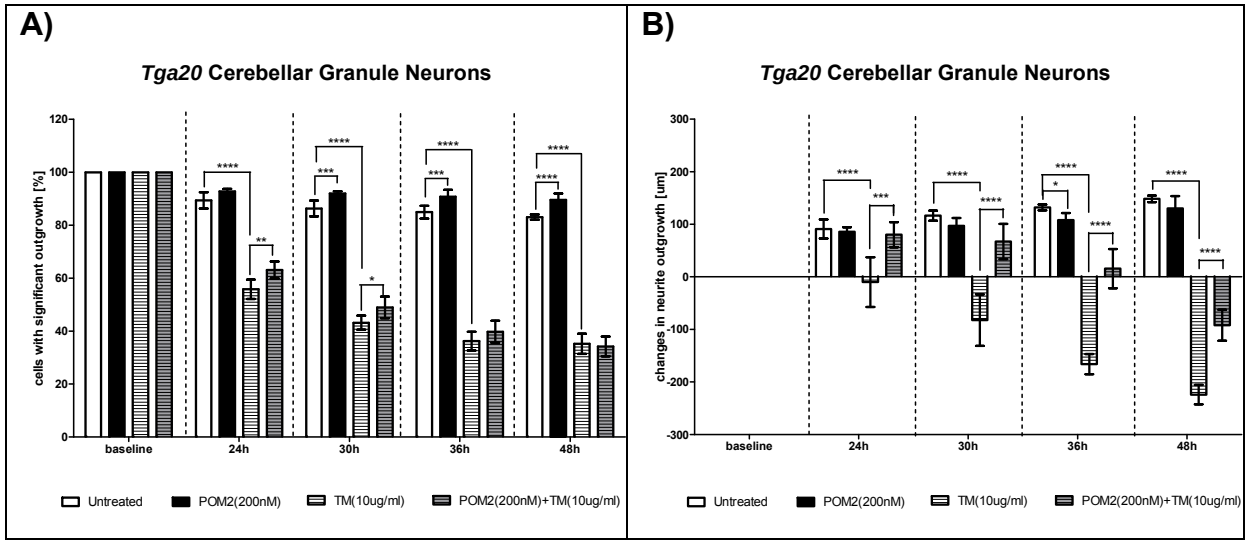


Figure 22: POM2 delays ER stress induced cell death in primary *tga20* cerebellar granule neurons

A)+B) 5 day old primary *tga20* granule neurons were exposed to POM2 (200 nM) 2 h before treatment with TM (10 ug/ml). The toxic effects of TM were ameliorated in cells pre-treated with POM2. Application of POM2 delays the reduction in the number of cells with significant outgrowth as well as neurite outgrowth in ER stress induced neurons. *Each error bar indicates the average SD of 5 technical replicates. The two-way ANOVA with Bonferroni post-test was used for this experiment (compared groups: Untreated-POM2, untreated-TM, TM-POM2+TM) ($p < 0.0001$ = ****, $p < 0.001$ = ***, $p < 0.01$ = **, $p < 0.1$ = *).*

6.5.1.2. The protective effect of POM2 is PrP^C dependent

To confirm that the protective effect of POM2 treatment against cellular stress/cell death is dependent on the expression of PrP^C, I performed the same experiment in ZH3 primary granule neurons that do not express PrP^C. POM2 was not able to induce significant changes in the cells, suggesting that the effect indeed is PrP^C dependent.

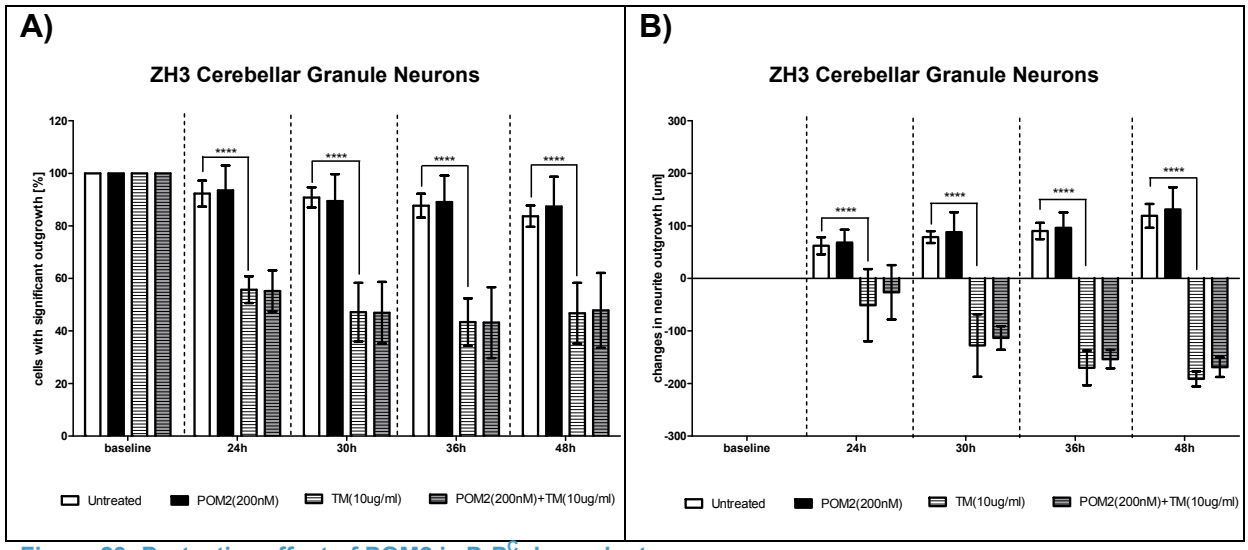


Figure 23: Protective effect of POM2 is PrP^C dependent
5 day old primary neurons of PrP^C knock out mice (ZH3) were pre-incubated with POM2 2h before the application of TM (200 nM). POM2 treatment had no influence on the cell number with significant outgrowth or on changes in neurite outgrowth. *Each error bar indicates the average SD of 5 technical replicates. The two-way ANOVA with Bonferroni post-test was used for this experiment (compared groups: Untreated-POM2, untreated-TM, TM-POM2+TM) (p < 0.001 = ****, p < 0.001 = ***, p < 0.01 = **, p < 0.1 = *).*

6.5.1.3. Investigation of translational changes after ER stress of POM2 pre-incubated cells

My preliminary data suggest that POM2 is able to delay neuronal toxicity triggered by ER stress. To address this observation in a human physiological context, iPSC derived human pan-neurons were used. In an initial approach, human neurons were pre-incubated for 2 h with POM2 before the application of TM (10 ug/ml). As a control, POM2 was inhibited with the addition of the PrP fragment P20 (Polymenidou et al., 2008). This peptide contains the binding region of POM2 and should prevent the interaction of POM2 with PrP^C. After 24 h the protein was collected and changes in the expression level of several proteins were analyzed.

Interestingly, PrP^C levels are highly upregulated upon induction of ER stress as previously shown (Dery et al., 2013; Misiewicz et al., 2013). However, no changes in the PrP^C expression level upon POM2 treatment were detected (see Figure 24 A).

In Alzheimer's disease, changes in synaptic plasticity can be evoked via ER stress (Endres and Reinhardt, 2013; Duran-Aniotz et al., 2014). In particular, synaptic degradation is associated with the activation of Fyn through the coupling of Aβ oligomers with the mGluR5-PrP^C complex (Um and Strittmatter, 2013; Nygaard et al., 2014). There are also several indications that PrP^C leads to an activation of Fyn via

the plasma membrane protein caveolin-1 (Mouillet-Richard et al., 2000; Toni et al., 2006; Shi et al., 2013). It seems that the octapeptide-region of PrP^C is especially important for this interaction (Shi et al., 2013). This is the binding region of the POM2 antibody (Polymenidou et al., 2008) suggesting a potential role of POM2 in the Fyn pathway. Caveolin-1 and P-Fyn levels were analyzed to examine the impact of POM2 on Fyn signaling. However, there were no obvious changes after TM treatment and a POM2 specific effect could not be observed (see Figure 24 B, C).

The UPR caused by ER stress leads to an induction of ERK1/2 (mitogen-associated protein kinases (MAPK) p42/44) (Darling and Cook, 2014), which was identified as a downstream target of the Fyn pathway (Schneider et al., 2003). In prion infected mice (LaCasse et al., 2008) and cell lines, ERK1/2 is activated (Didonna and Legname, 2010; Pradines et al., 2013). As expected, human neurons treated with TM showed phosphorylation of ERK1/2; however, there was no indication that POM2 could prevent this (see Figure 25 D).

BiP is a master regulator of the UPR (Wang et al., 2009; Wang et al., 2010b). It has been shown that the disease associated mutant (Q217R) (Jin et al., 2000) as well as the flexible tail of PrP^C (FTgpi) interacts with BiP (Dametto et al., 2015). Since PrP^C interacts with BiP, I checked the regulation pattern of BiP upon POM2 treatment. BiP was upregulated in neurons treated with TM; however, POM2 could not prevent the induction of protein expression. Alternatively, POM2 pre-incubated with P20 and P20 alone resulted in a slightly decreased signal (see Figure 25 E).

The transcription factor CCAAT-enhancer-binding protein homologous protein (CHOP) is significantly upregulated after the induction of ER stress (Cheng and Yeh, 2003; Reimertz et al., 2003) and CHOP signaling seems to be implicated in many PMDs, for example upregulation of CHOP was observed in ALS and Alzheimer's patients and mouse models (Milhavet et al., 2002; Ito et al., 2009; Lee et al., 2010; Sasaki, 2010). While the transduction of CHOP in Huntington disease patients was increased (Vidal et al., 2012), no changes were observed on the protein level (Carnemolla et al., 2009). A significant increase in the expression levels of CHOP was triggered upon treatment with the ER stressor TM. Pre-incubation of the cells with POM2 did not lead to a decrease in the CHOP signal (see Figure 25 F).

At least for the proteins tested, POM2 did not reveal any beneficial effect. However, based on the morphometric analysis POM2 was able to delay the neurite degeneration induced by ER stress. It is suggested that PrP^C can promote neurite

outgrowth via interaction of different proteins such as laminin (Graner et al., 2000b; Graner et al., 2000a; Coitinho et al., 2006; Beraldo et al., 2011; Santos et al., 2013), vitronectin (Hajj et al., 2007), NCAM (Santuccione et al., 2005) stress-inducible protein-1 (STI-1) (Lima et al., 2007; Santos et al., 2013) and even PrP^C itself (Chen et al., 2003). On the other hand inhibition of neurite outgrowth was observed by the interaction of PrP^C with contactin-associated protein (Caspr) (Devanathan et al., 2010). Thus interaction of POM2 with PrP might interfere with neuritogenesis signaling.

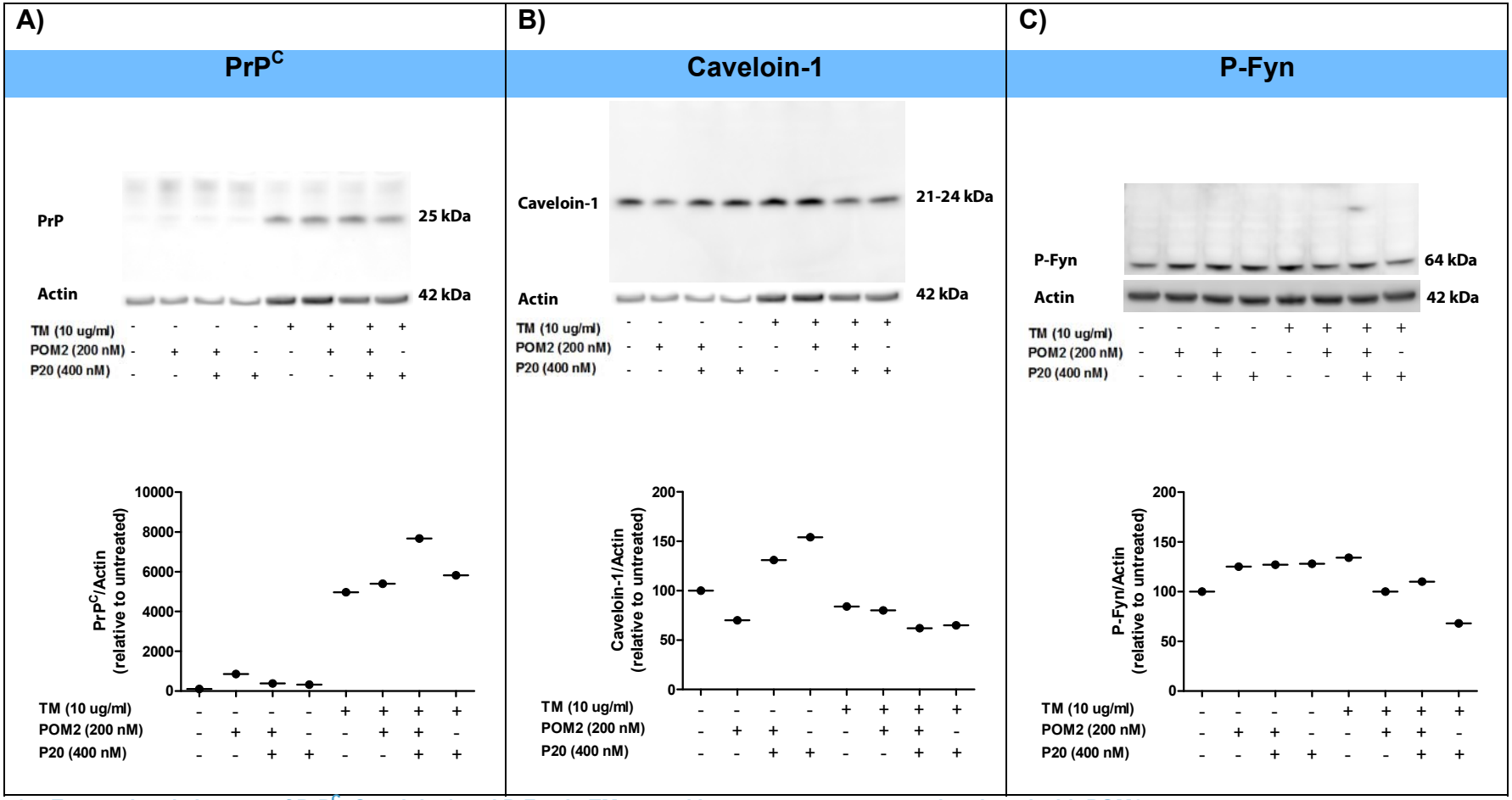


Figure 24: Expressional changes of PrP^C, Caveloin-1 and P-Fyn in TM treated human pan-neurons pre-incubated with POM2

iPSC derived human pan-neurons were pre-incubated with POM2 (200 nM), P20 (400 nM) and POM2 (200 nM) plus P20 (400 nM) 2h before induction of ER stress with TM (10 ug/ml). After 24h the protein was isolated and expression levels of PrP^C, P-Fyn and Caveloin-1 were examined *(1 replicate was used per condition)*.

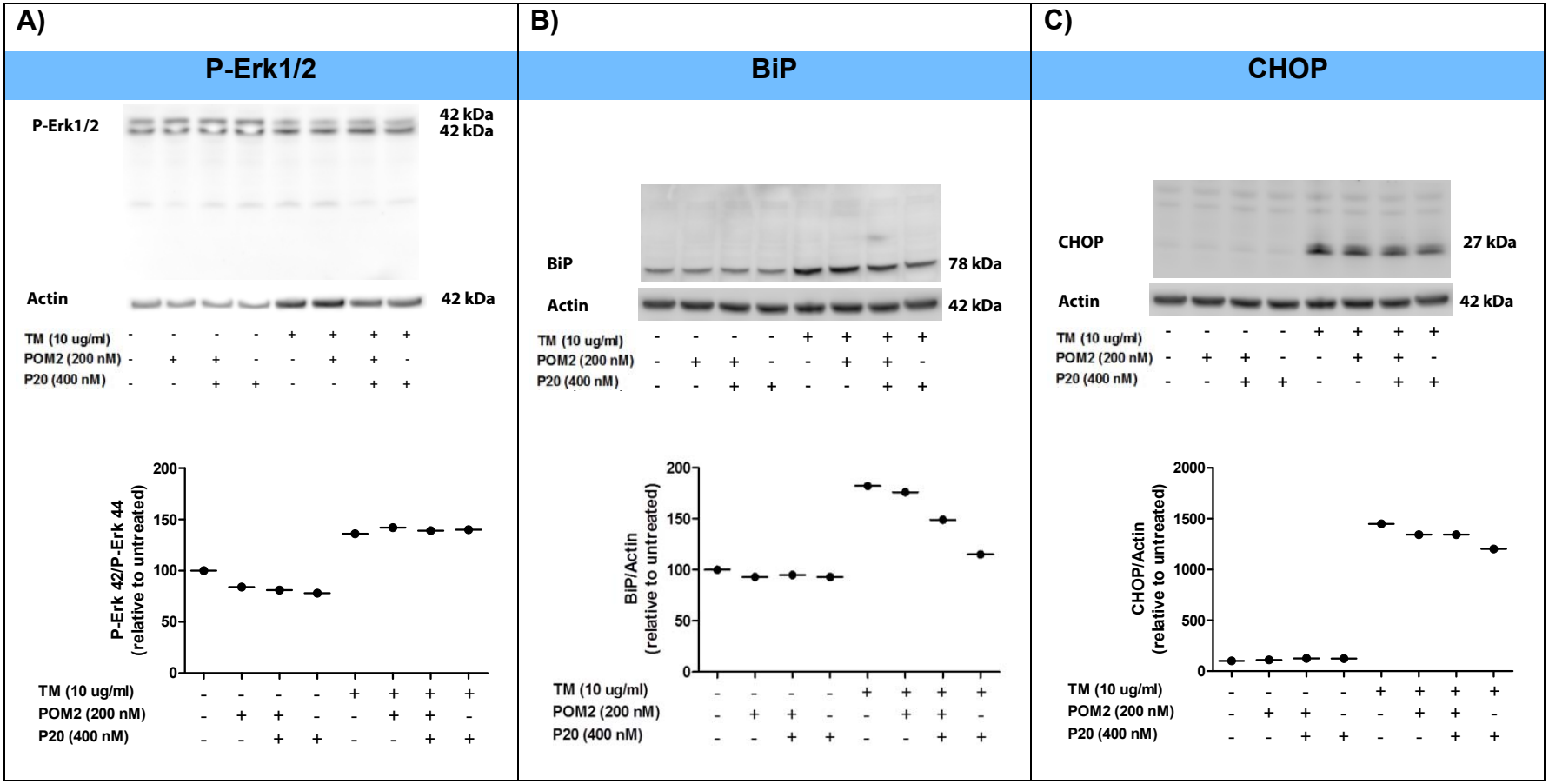


Figure 25: Expressional changes of P-Erk1/2, BiP and CHOP in TM treated human pan-neurons pre-incubated with POM2

iPSC derived human pan-neurons were pre-incubated with POM2 (200 nM), P20 (400 nM) and POM2 (200 nM) plus P20 (400 nM) 2h before induction of ER stress with TM (10 ug/ml). After 24h the protein was isolated and expression levels of P-Erk1/2, CHOP and BiP were examined *(1 replicate was used per condition)*.

I changed the treatment conditions in order to reveal translational changes upon POM2 treatment in iPSC derived human neurons that have undergone ER stress. Based on previous data obtained from primary cerebellar granule neurons treated with TM, it appears that POM2 is delaying cell death rather than inducing a complete rescue. Therefore, I decided to assess the time course of the expression level of selected proteins involved in ER stress.

The experimental settings were adjusted in order to improve the effect caused by POM2. In particular, the incubation time of POM2 before the stressor treatment was decreased from 2 to 0.5 h to reduce the amount of antibody that is degraded or endocytosed at the time the stressor is added. Furthermore, a lower concentration of TM was applied to decrease the toxic effect and the ER stressor Thapsigargin (TH) was applied. TH leads to a depletion of calcium in the ER and an upregulation of extra-cellular calcium via the inhibition of the sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) (Treiman et al., 1998). After stressor treatment, the protein was collected at 3, 6 and 9 h time points.

The levels of PrP^C, BiP and CHOP were rechecked. In addition, the expression levels of the transcription factor ATF4 were analyzed. Studies of Galehdar *et al.* in primary mouse cortical neurons reveal that after TM and TH application, ATF4 is markedly upregulated and the depletion of ATF4 leads to significantly increased survival (Galehdar et al., 2010). In addition, enhanced translation of ATF4 was shown in RML inoculated and POM1 treated brain slices (Herrmann et al., 2015), as well as in Alzheimer's disease patients and mouse models (Ma et al., 2013; Baleriola et al., 2014; Wei et al., 2015).

In the following figures, the western blots at the 3h, 6h and 9h time points and the time course of the protein level for the selected proteins are depicted.

At the 6h time point an increase in the PrP^C levels only in the sample treated with 500 nM TH was observed while pre-incubation of POM2 and POM2 plus P20 block this effect. However, for other time points, no conclusive pattern could be observed (see Figure 26).

The protein level of BiP for each time point is lower in the POM2 or POM2 plus P20 treated cells than in the control. After TM treatment no upregulation of BiP could be observed for the 3 and 6h time point and after 9h the BiP levels in the untreated samples are higher than the control (see Figure 27). This suggests that TM did not

cause ER stress or the ER was too weak to activate the BiP after this time frame. After 3h for both concentrations of TH, the levels of BiP were increased. At this time point POM2 and POM2 plus P20 could decrease the BiP signal. Given that P20 should prevent the effect of POM2, the observed downregulation of BiP in this control samples could only be attributed to insufficient blocking of POM2 with P20. The lower concentration of TH caused a stronger increase in the BiP signal at the 3h time point, while at the 6h and 9h time points, no dramatic difference was observed between the BiP levels. In particular, at 6h the pre-incubation of POM2 in the 250 nM TH treated neurons revealed a significant decrease of BiP that might be due to a protective effect of POM2. However, further experiments are needed to confirm these preliminary data.

For the TH treated samples, a clear upregulation of ATF4 and CHOP could be observed for all time points, whereas the concentration of TM used was not able to induce upregulation of these UPR markers. At 3 and 6 hours after TH treatment, the translational level of ATF4 and CHOP was lower in the POM2 /TH treated cells than in the neurons treated only with TH. At the 9h time point, the signal of ATF and CHOP also increased in TH/POM2 treated samples (see Figure 28 and Figure 29). These results suggest a delay in UPR upregulation caused by POM2 treatment. Again, more replicates are needed for a more conclusive statement.

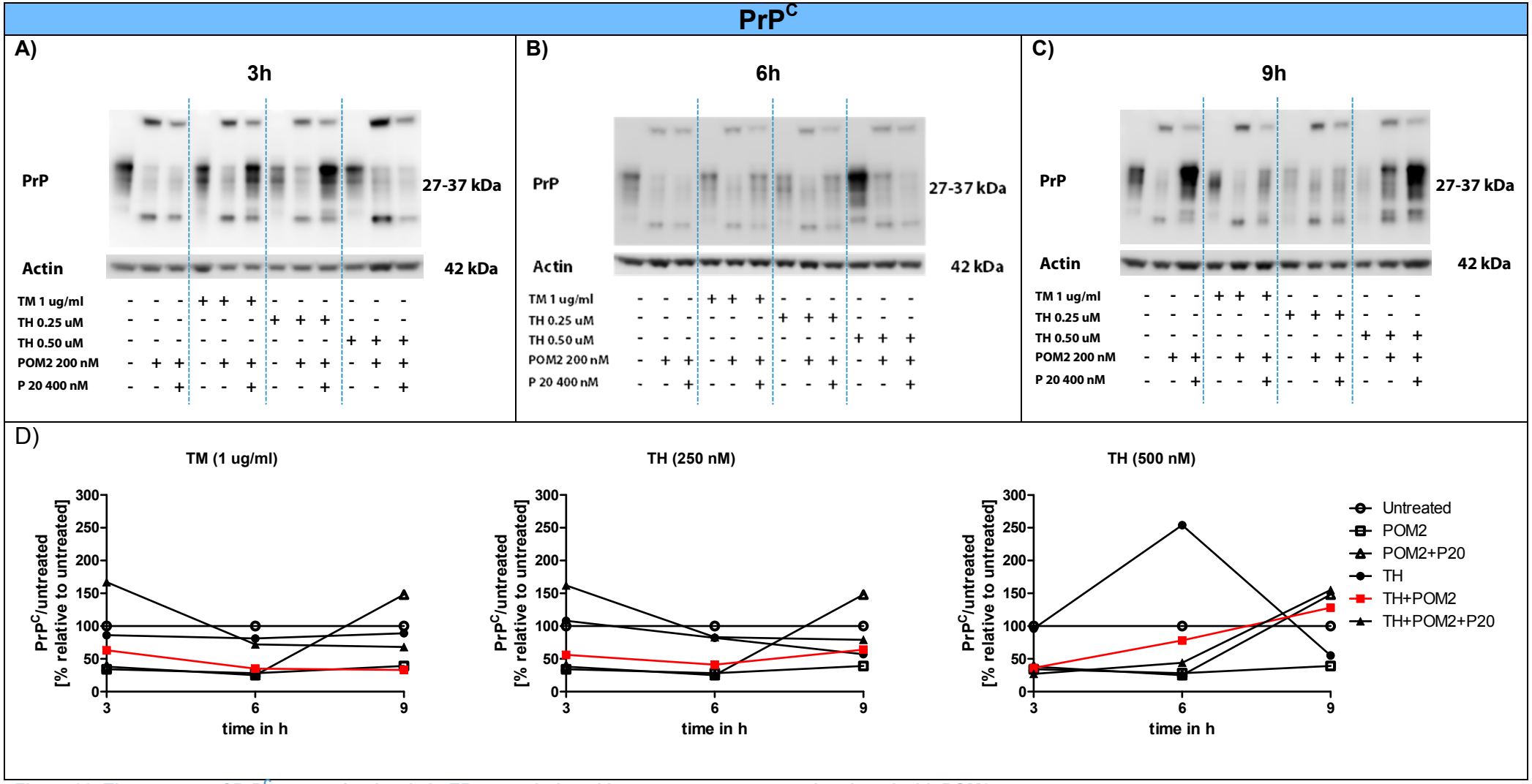


Figure 26: Time course of PrP^C expression levels in ER stress induced human pan-neurons pre-incubated with POM2

A)- D) iPSC derived human pan-neurons were pre-incubated with POM2 (200 nM), P20 (400 nM) and POM2 (200 nM) plus P20 for 0.5 hours before the treatment of TM (1 ug/ml) or TH (250 nM or 500 nM). After 3h, 6h and 9h the protein was collected and the translational level of PrP^C determined *(per condition, 1 replicate was used)*. A)-C) The bands at 25 and 50 kDa are derived from the heavy and the light chain of the POM2 antibody.

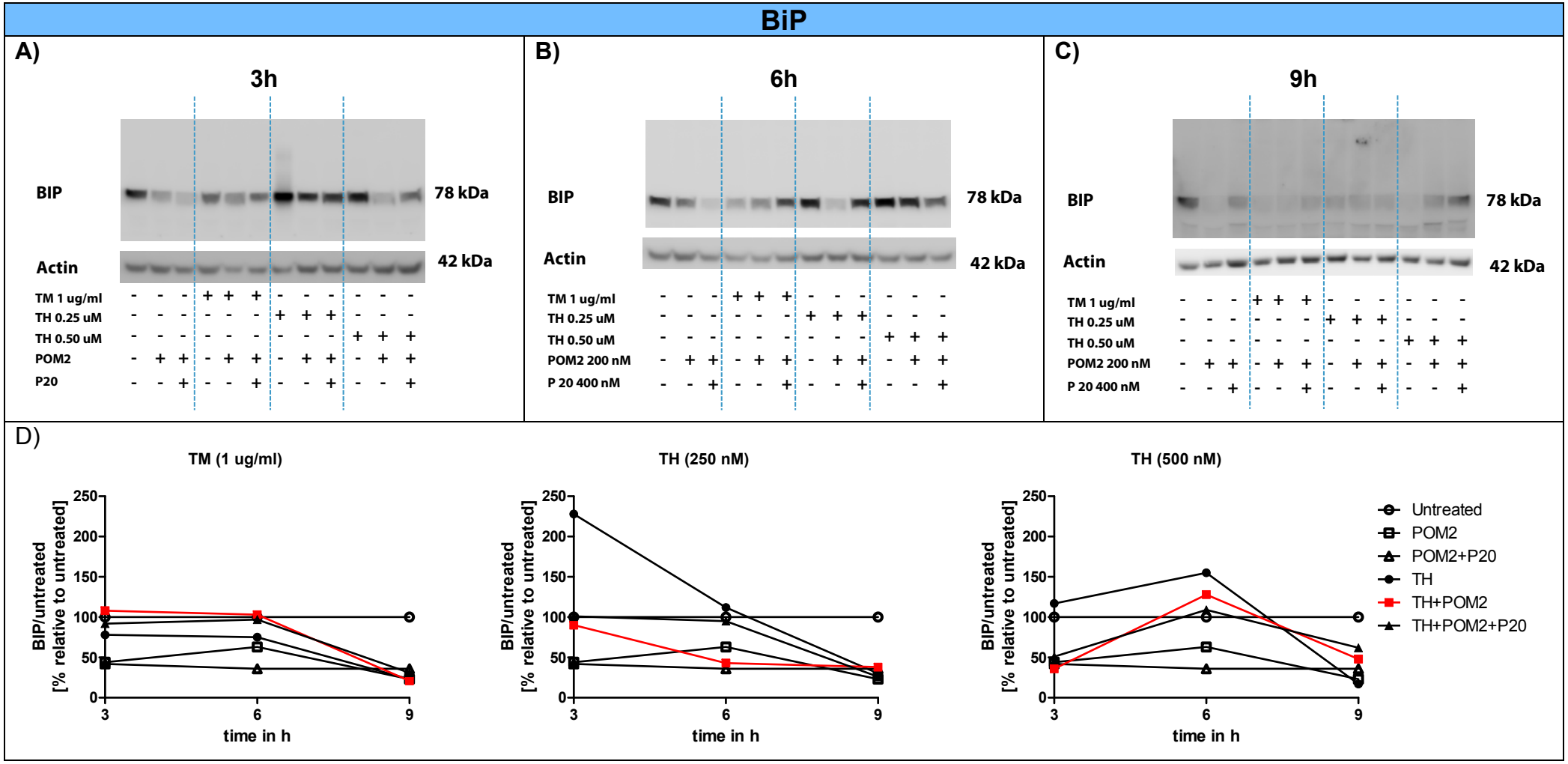


Figure 27: Time course of BiP expression levels in ER stress induced human pan-neurons pre-incubated with POM2

A)- D) iPSC derived human pan-neurons were pre-incubated with POM2 (200 nM), P20 (400 nM) and POM2 (200 nM) plus P20 for 0.5 hours before the treatment of TM (1 ug/ml) or TH (250 nM or 500 nM). After 3h, 6h and 9h the protein was collected and the translational level of BiP determined *(per condition, 1 replicate was used)*.

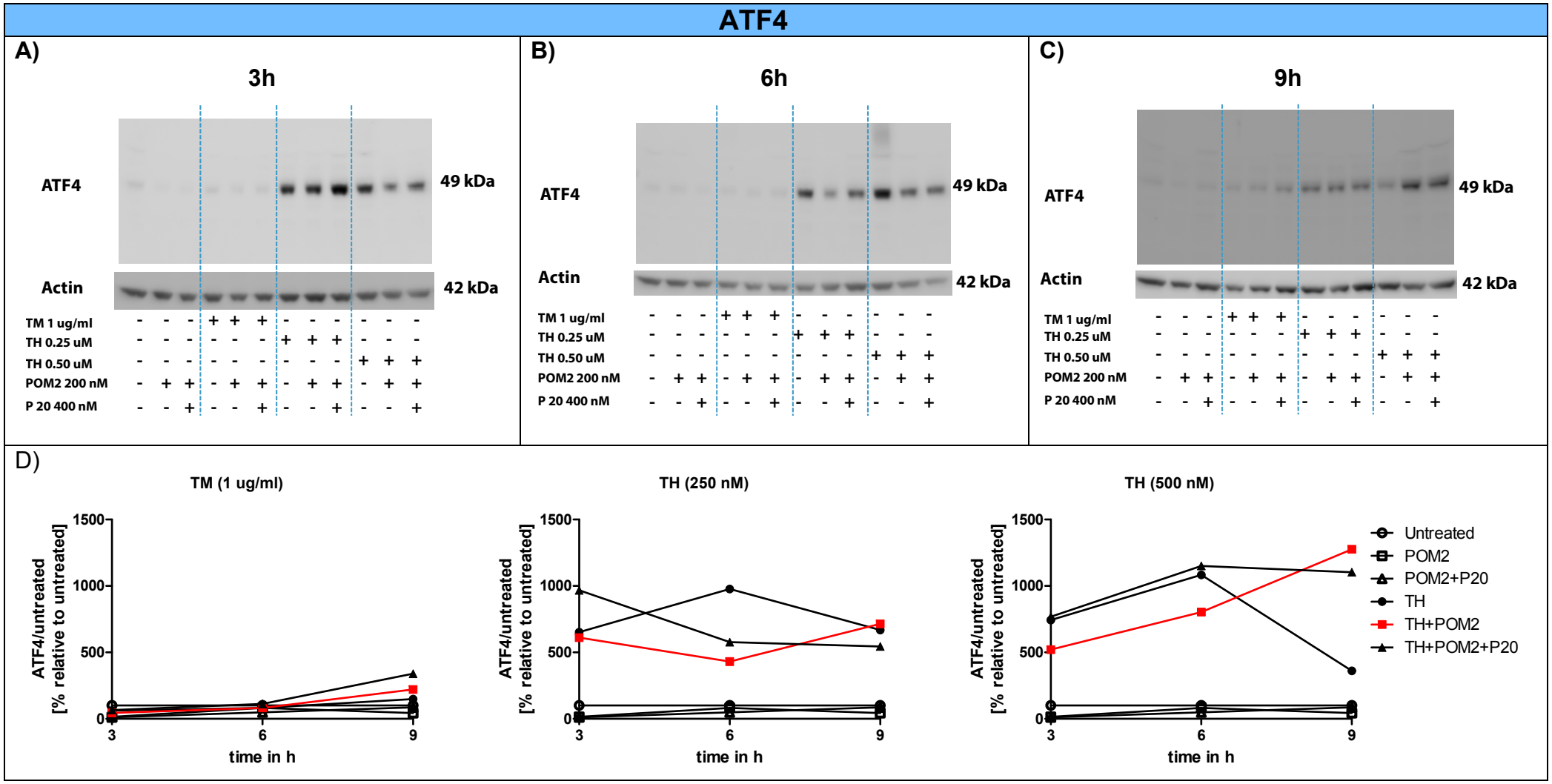


Figure 28: Time course of ATF4 expression levels in ER stress induced human pan-neurons pre-incubated with POM2

A)- D) iPSC derived human pan-neurons were pre-incubated with POM2 (200 nM), P20 (400 nM) and POM2 (200 nM) plus P20 for 0.5 hours before the treatment of TM (1 ug/ml) or TH (250 nM or 500 nM). After 3h, 6h and 9h, the protein was collected and the translational level of ATF4 determined *(per condition, 1 replicate was used)*.

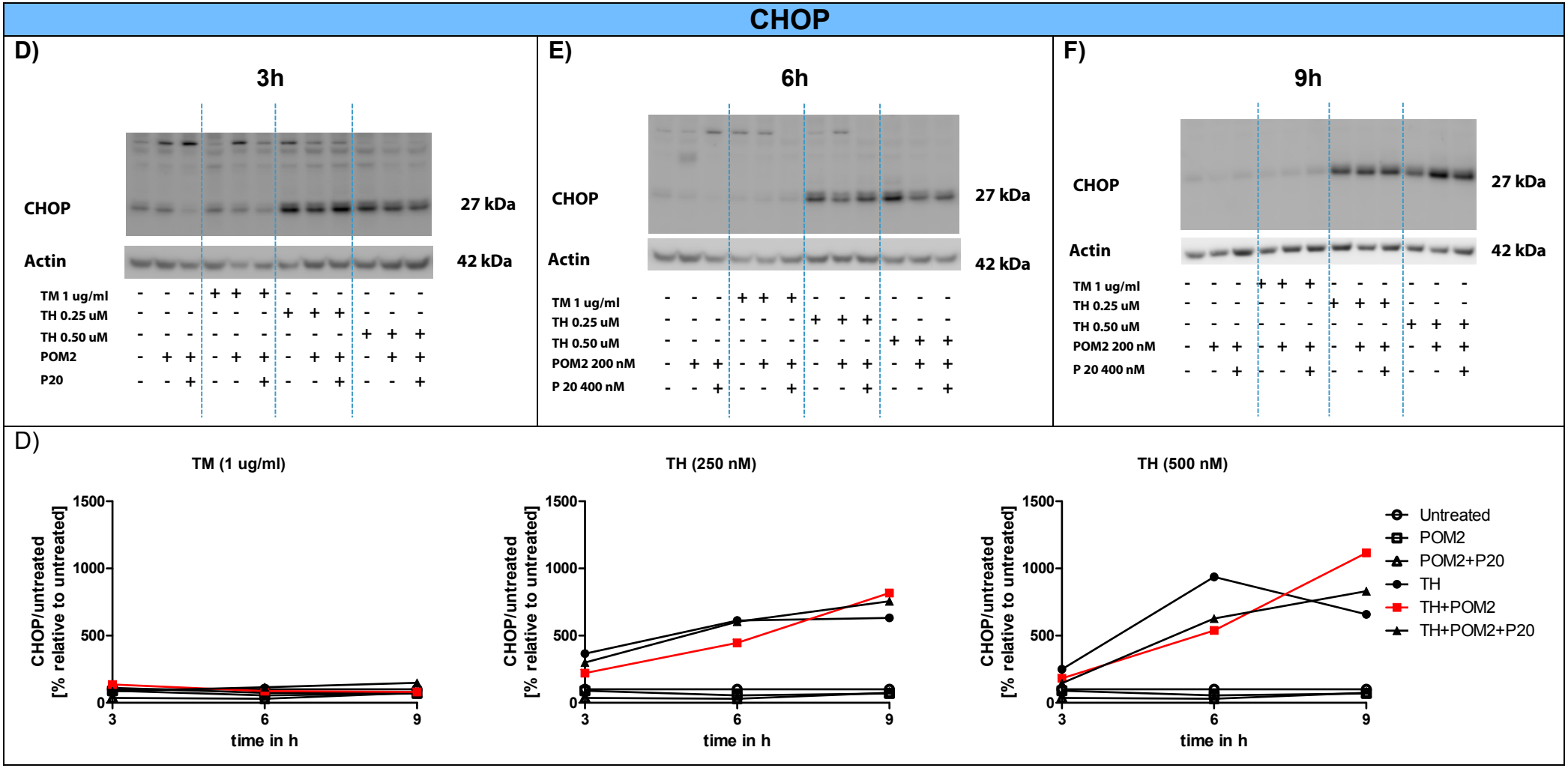


Figure 29: Time course of CHOP expression levels in ER stress induced human pan-neurons pre-incubated with POM2

A)- D) iPSC derived human pan-neurons were pre-incubated with POM2 (200 nM), P20 (400 nM) and POM2 (200 nM) plus P20 for 0.5 hours before the treatment of TM (1 ug/ml) or TH (250 nM or 500 nM). After 3h, 6h and 9h, the protein was collected and the translational level of CHOP determined *(per condition, 1 replicate was used)*.

6.5.2. In vivo models to test the therapeutic effect of POM2

To evaluate if PrP^C can act as a general cell death transducer in different neurodegenerative diseases, the therapeutic potential of POM2 should be evaluated in several animal models of neurodegenerative diseases.

6.5.2.1. Establishment of an application method for POM2 in in vivo experiments

Injection of POM2 in *tga20* mice was shown to be lethal (Polymenidou, unpublished data). *In vitro* data suggest that POM2 leads to lysis of red bloods cells in a PrP^C dependent manner through complement activation (Sonati et al., 2013).

If POM2 would indeed cause hemolysis, in vivo application of the POM2 holo antibody would not be possible. Therefore, the intravenous injection of the POM2 holo antibody in mice was repeated to exclude any contamination in the antibody preparation had caused this effect in previous results.

The POM2 antibody was injected into three different genotypes expressing different levels of PrP^C to evaluate if POM2 induces hemolysis in a PrP^C dependent manner. In particular, ZH3 (PrP^C knock out), C57BL/6J (PrP^C wildtype) and *tga20* mice (PrP^C overexpressing) were intravenously injected with 100 ul antibody solution (concentration = 1ug/ul) of the POM2 holo antibody or the negative control mouse IgG. After 1h blood was collected in BD microtainer SST^{TM} tubes for serum collection. In addition, 1 day before the experiment and 5 min after injection of the antibody solution, blood was drawn into EDTA coated tubes.

The serum of *tga20* mice injected with POM2 showed a much stronger red coloration than other samples, which suggested that hemolysis had been induced (see Figure 30).

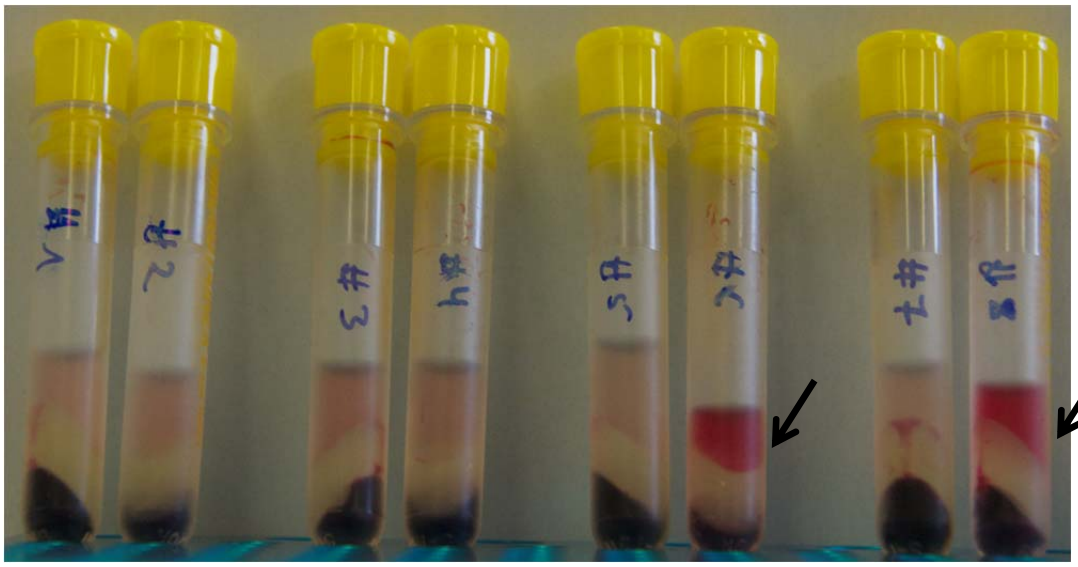


Figure 30: POM2 causes hemolysis in PrP^C overexpressing mice after 1h in vivo
ZH3 (PrP^C knock out; #1-2), C57BL/6J (PrP^C wildtype; #3-4) and *tga20* mice (PrP^C overexpressing; #5-8) were injected with 100 ul (1 ug/ul) antibody solution of POM2 or IgG. After 1h blood was collected in BD microtainer SST^{TM} tubes to achieve serum. Only the serum of PrP^C overexpressing mice (# 7 and 8) showed an intensive red color suggesting that hemolysis had occurred.

For quantification of the hemoglobin concentration in serum, the toluidine assay was used (Goyal and Basak, 2009). The principle of this assay as follows: hemoglobin converts hydrogen peroxide to water and nascent oxygen (Goyal and Basak, 2009). The nascent oxygen then oxidizes yellow ortho-toluidine to a green blue reaction product (Goyal and Basak, 2009). The kinetics of the reaction is monitored at an absorbance of 630 nm, whereby the conversion rate to ortho-toluidine is directly proportional to the haemoglobin concentration (Goyal and Basak, 2009). The quantification of the hemoglobin in the serum indicates that POM2 induces hemolysis in mice overexpressing PrP^C.

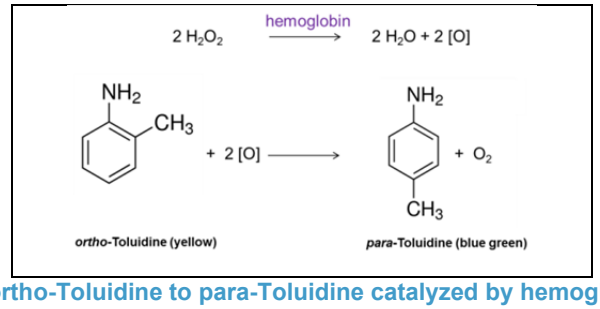


Figure 31: Conversion of ortho-Toluidine to para-Toluidine catalyzed by hemoglobin
Hemoglobin converts hydrogen peroxide to water and nascent oxygen, which in turn oxidizes ortho-toluidine to para-Toluidine.

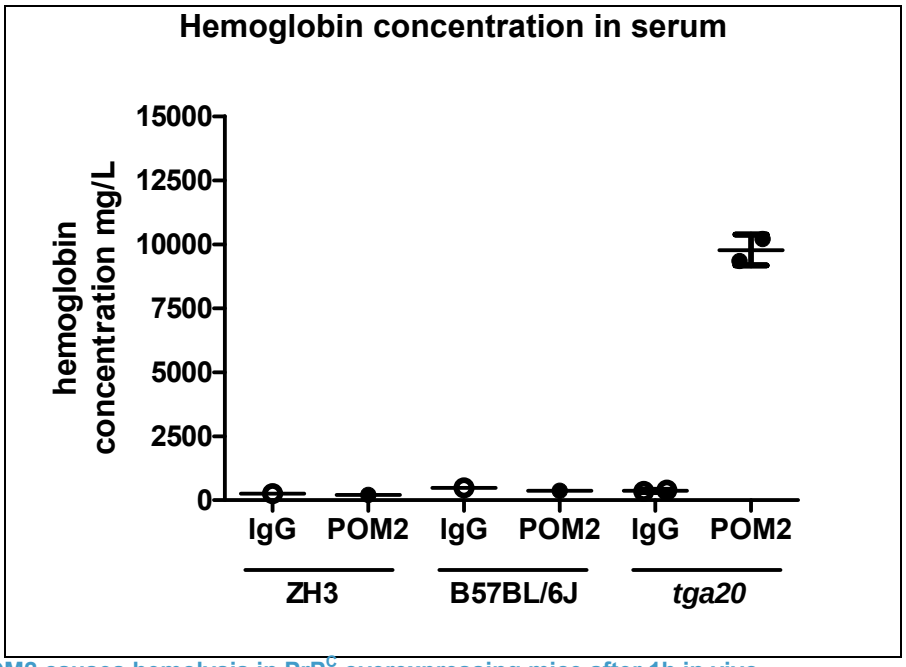


Figure 32: POM2 causes hemolysis in PrP^C overexpressing mice after 1h in vivo
PrP^C depleted (ZH3), PrP^C wild type (B57BL/6J) and PrP^C overexpressing (tga20) mice were intravenously injected with 100 ul IgG or POM2 (1 ug/ul) solution. After 1h blood was collected and serum isolated. The hemoglobin concentration in the serum was determined with the o-Toluidine assay. *For the ZH3 and B57BL/6J samples, one mouse was injected per antibody solution, and for the tga20 samples, two mice were injected per antibody solution.*

6.5.2.2. Generation of an Adeno associated virus expressing scPOM2

The POM2 holo antibody causes hemolysis and cannot be administered intravenously. It is assumed that hemolysis is mediated by complement activation. Antibodies can cause complement activation via their Fc region (Noris and Remuzzi, 2013; Melis et al., 2015). Therefore, it was decided to use the scFv POM2 for the planned *in vivo* studies. The use of the scFv antibody fragment is hampered by the fact that these fragments are not very stable *in vivo* (Worn and Pluckthun, 2001). To circumvent this issue, an Adeno associated virus (AAV) that allows the continuous expression of the antibody fragment *in vivo* was generated and the virus was prepared in collaboration with Prof. Dr. Bernard Schneider. Helper plasmids for the virus production were provided by Bernard Schneider and plasmids containing the scFv POM2 were designed and made by Dr. Vijay Chandrasekar. Since the virus will be applied to animal models of neurodegenerative diseases, a general promoter (cytomegalovirus (CMV)) was used for the scFv POM2 expression. Compared to a neuron specific promoter, this has the advantage that significantly more cells can be transduced and neuronal cell loss will not heavily affect the expression of scFv POM2. The Kozak sequence will be recognized by the ribosome and leads to an

enhanced translation initiation (Nakagawa et al., 2008). The BM40 signal peptide sequence ensures the secretion of scFv POM2, which allows a wide spread distribution of the therapeutic agent. The attachment of the His-Tag enables detection of the scFv POM2 after secretion. The internal ribosome entry side (IRES) sequence allows the initiation of translation within the mRNA (Komar and Hatzoglou, 2011; Thompson, 2012) and is used to express the fluorescent marker tdTomato to track the expressing of the antibody.

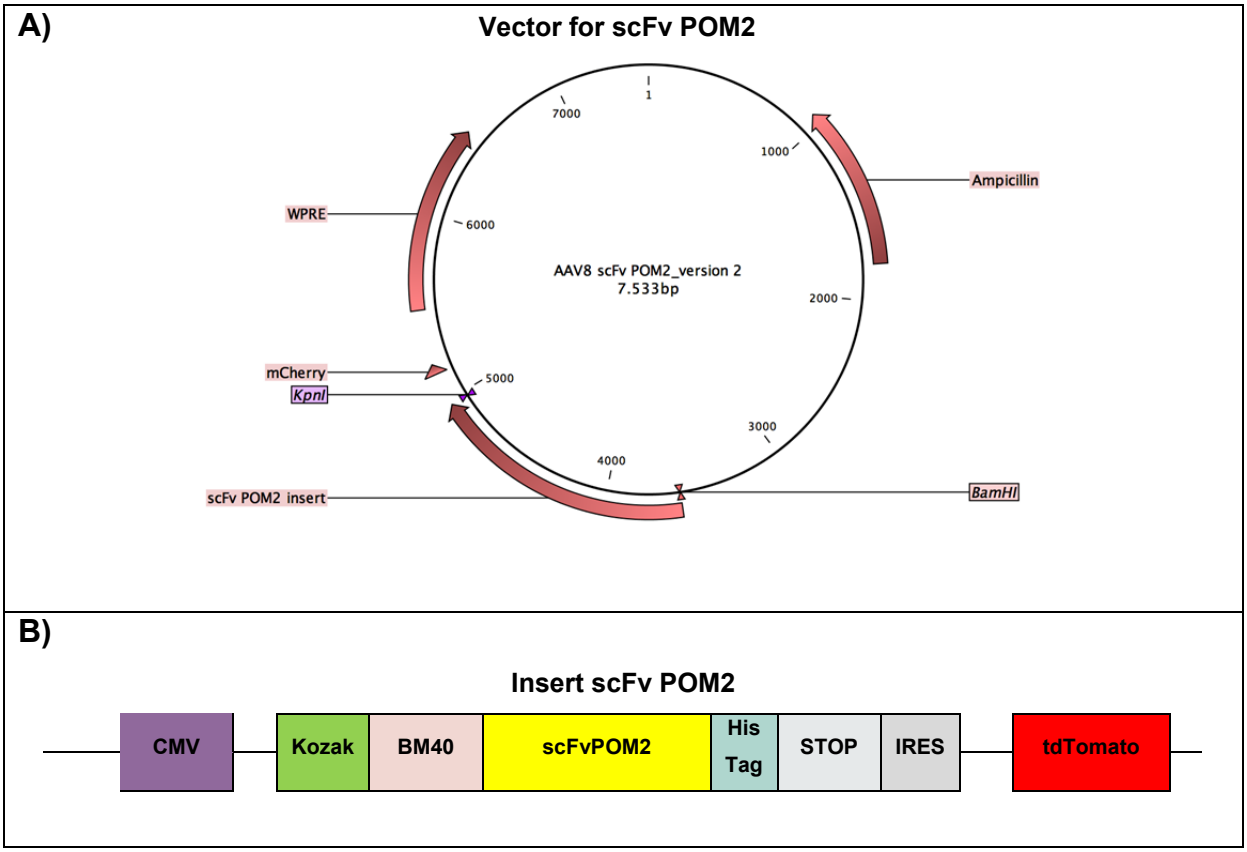


Figure 33: scFv POM2 plasmid for AAV production
A) The vector pAAV-CAG-tdTomato was cut on the BamHI and KpnI position. B) The scFv POM2 insert was cloned into the linearized vector.

6.5.2.2.1. Transduction efficiency of Adeno associated virus ex vivo

Virus production was done at the lab of Prof. Bernard Schneider. In a first trial it was planned to use the viral expression of scFv POM2 in a model of photoreceptor induced toxicity in the mouse eye detailed under 6.5.2.2.2. Therefore, it was decided to use the AAV8 serotype for our experiments. This serotype is especially suitable for a high transduction rate in the mouse retina.

AAV8 is not suitable for transducing cell lines or primary cell cultures according to Prof. Bernard Schneider. Therefore, we tested the expression of the virus in cerebellar organotypic brain slices of C57BL/6 mice.

For long term survival of organotypic brain slices, cultivation at an air/liquid interface is crucial (Gahwiler, 1981; Gahwiler et al., 2001). This can be achieved either by culturing of the slices on semiporous membranes (Gahwiler, 1981) or by rotating them in a roller-tube filled with a small amount of medium (Gahwiler et al., 2001) (see Figure 34). We expected that the transduction potential of AAV is higher in roller-tubes based on the continuous movement of the media over the slices. In addition, live imaging is less cumbersome, since the coverslip can be just placed under the microscope and cutting of the membrane is not necessary, which might otherwise destroy the slices.

In brief, two cerebellar slices were embedded in a plasma clot on a glass coverslips and medium containing AAV8 expressing scPOM2 were added. Control slices were treated with AAV6 expressing GFP under the synapsin promoter. The use of a different serotype was based on the fact that the AAV6 was already shown in the lab to transduce slices efficiently. For the transduction of mouse brain slices with AAV8 no significant results have been published. For the AAV6 at titer of 10^8 and 10^9 and for the AVV8 a titer of 10^9, 10^{10} and 10^{11} was used. After 1h of incubation at 4 °C the tubes were put into the rotating wheel within the incubator and viral expression was tested after 10 days.

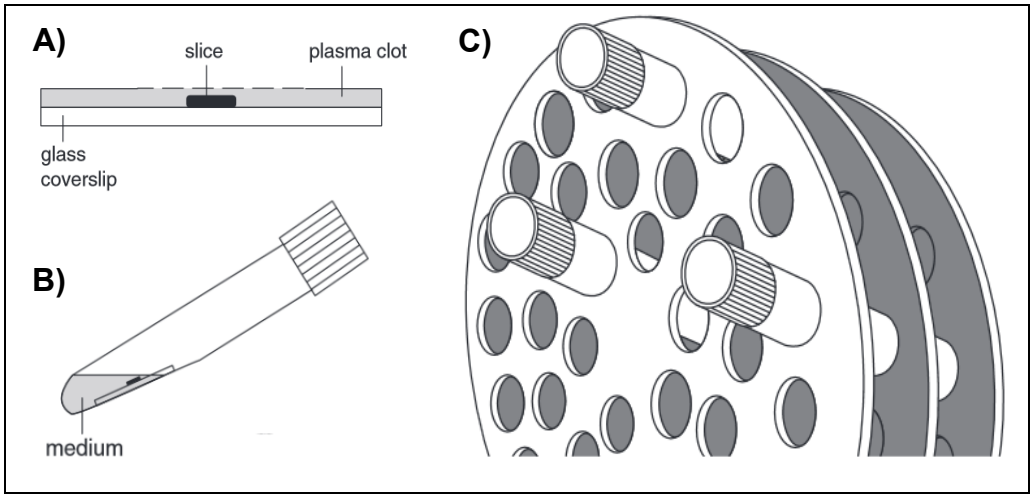


Figure 34: Cultivation of brain slices in roller tubes
A) Glass coverslips were mounted with brain slices, which were imbedded in a plasma clot. B) These coverslips were then put in a roller tube with a small amount of medium, a plastic culture tube that has a flat-sided bottom. C) For cultivation of the brain slices, the tubes were put in a special incubator containing a slow rotating wheel that allows the alternation of aeration and nutrition of the slice (images modified (Gahwiler et al., 2001)).

10 day after infection, significant transduction of all slices with different titers of AAV8 scFv POM2 or AAV6 GFP could be observed (see Figure 35). In general, AAV6 seem to infect mostly the granule layer, while the AAV8 mainly transduces the molecular layer.

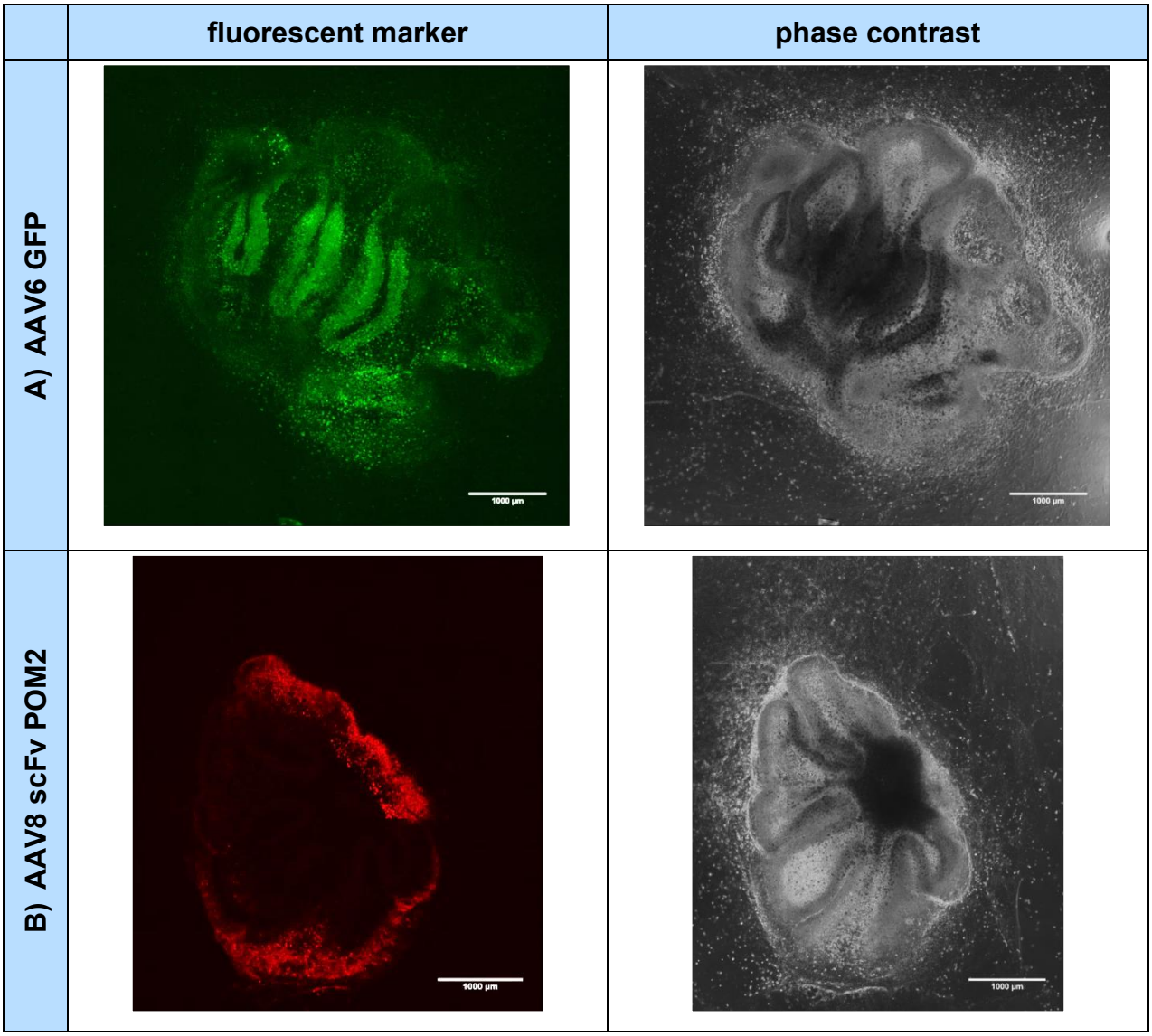


Figure 35: Cerebellar brain slices transduced with AAV8
Cerebellar brain slices were transduced with A) AVV6 GFP or B) AAV8 scFv POM2 (titer = $e10^9$) and cultured in roller-tubes. 10 days after transduction slices were imaged. The AAV6 infected mostly the granule layer while the AAV8 revealed the strongest signal in the periphery.

I have shown that the AAV8 scFv POM2 is able to transduce slices. The secretion levels of scFv POM2 are also important for the success of our studies. Therefore, to quantify scFv POM2 levels, the medium of the infected slices were collected and scFv POM2 expression was assessed by ELISA with the help of Rita Moos. Since

scFv POM2 design has a His-Tag, this was used for Biotin-Streptavidin-HRP detection of the scFv (see Figure 36).

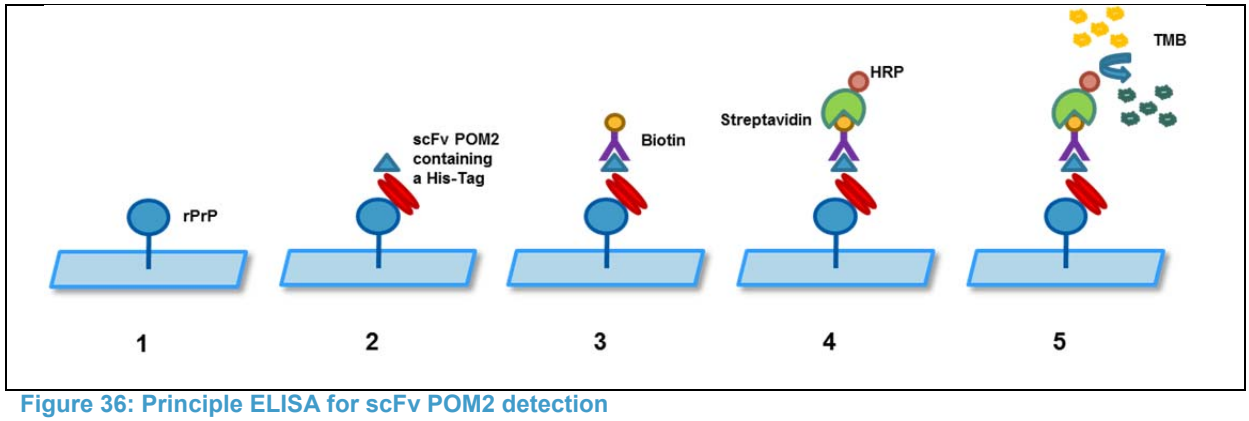


Figure 36: Principle ELISA for scFv POM2 detection
Microtiterplates were coated with rPrP (1) and medium containing scFv POM2 were added (2). The scFv POM2 contained a His-Tag. This was used for binding of a biotin conjugated antibody (3). Streptavidin coupled with horse radish peroxidase (HRP) were binding to biotin (4). The addition of the HRP substrate 3,3′,5,5′-tetramethylbenzidin (TMB) allowed the indirect detection of the antibody (5).

Three different titers of AAV8 scFv POM2 were used. The expression of scFv POM2 was dependent on the titer. As expected, the slices not infected with virus or transduced with the control AAV6 GFP revealed no specific signal. As a positive control, scFv POM2 produced in bacteria was used. However, the detected concentration was higher than the applied one. The explanation for that observation might be a calculation error or the fact that the titration curve was solved in TopBlock and the scFv POM2 was diluted in slice culture media.

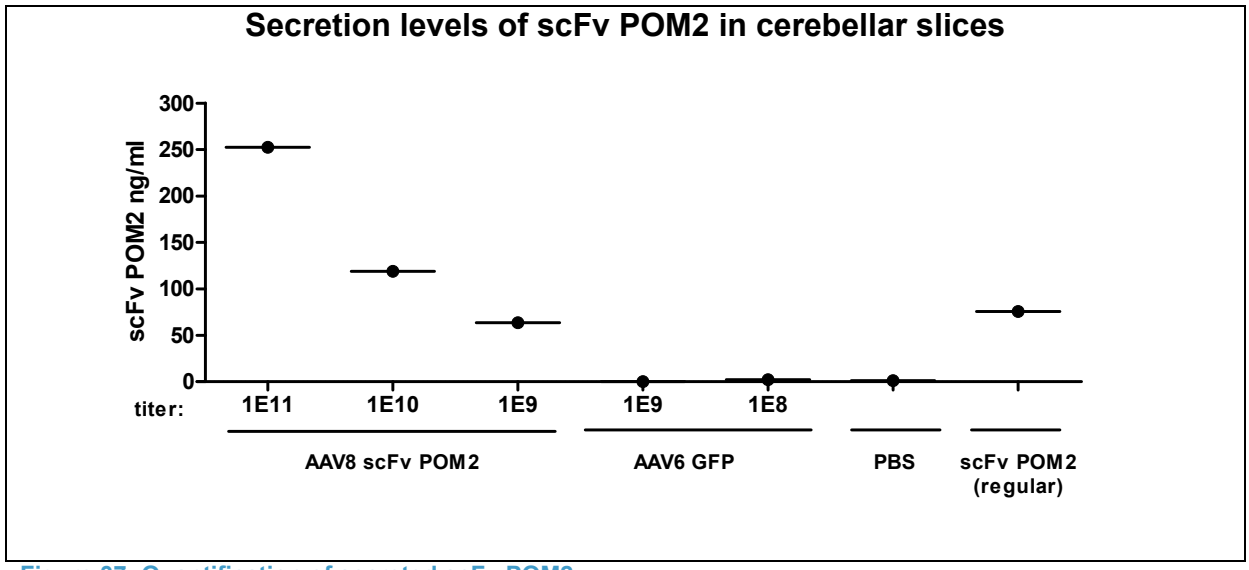


Figure 37: Quantification of secreted scFv POM2
On day 1 cerebellar slices of C57BL/6 mice were infected with AAV8 scFv POM2 (titer: 1e^9, 1e^{10}, 1e^{11}) and AAV6 GFP (titer: 1e^8, 1e^9) or left untreated (PBS). 7 days after medium change (day 9-16), the medium was collected and scFv POM2 levels were detected via ELISA. Only AAV8 scFvPOM2 transduced slices efficiently and expresses the antibody in a titer dependent manner. The additional control, scFv POM2, which was produced in bacteria, gave a convincing signal. The explanation for that observation might be a calculation error or the fact that the titration curve was solved in TopBlock and the scFv POM2 was diluted in slice culture media.

To achieve a therapeutic effect after POM2 treatment, a certain amount of scFv POM2 must be secreted. Therefore, the molarity of scFv POM2 per slice was calculated. Recently it was shown that the incubation of 6-10 slices with 335 nM (34 to 56 nM/slice) of the holo POM2 antibody counteracts the neuronal loss in RML infected slices. The transduction of the slices with AAV8 scFv POM2 give values from 113 to 451 nM scFv POM2/slice. Therefore we assume that this concentration is sufficient for therapeutic treatments, despite the fact that scFv fragments are less stable than holo antibodies (Worn and Pluckthun, 2001).

Table 9: Amount of secreted scFv POM2 in AAV8 scFV transduced slices

virus	titer	final ng/ml	nMol/two slices	nMol/slice
scFv POM2 AAV8	1^{e11}	253	902	451
scFv POM2 AAV8	1^{e10}	119	425	212
scFv POM2 AAV8	1^{e9}	64	227	113

6.5.2.2.2. Therapeutic effect of POM2 against photoreceptor-toxicity in the mouse eye

To check if PrP^C can indeed act as a death sensor in neurodegenerative diseases, the protective potential of the AAV8 expressing scFv POM2 should be tested in animal models of neurodegenerative diseases. However, the evaluation of scFv POM2 as a therapeutic tool in mouse models will likely prove to be quite time consuming, because it would mandate behavioral studies and the specific handling of

animal models with specific neurodegenerative phenotypes. In addition, these studies are quite expensive. Therefore, we decided to initially test the protective potential of the scFv POM2 antibody in a simpler *in vivo* model system. The induction of photoreceptor apoptosis by light in the mouse eye seems to represent an appropriate system. In prion inoculated mice and hamster, apoptosis mediated neuronal loss in the retina could be detected (Hogan et al., 1981; Kozlowski et al., 1982). Thus PrP^C expression in the eye seems to play an important role in mediating cell death in neuronal cells in the retina after prion infection. If PrP^C is a general cell death transducer, it is likely that it is also involved in mediating neurodegeneration induced by phototoxicity. Another advantage of this model system is the fast readout with results obtained in less than one month.

In collaboration with Prof. Dr. Christian Grimm, the protective potential of scFv POM2 against retina degeneration caused by phototoxicity was tested. For this experiment wild type BALB/c mice from Harlan and PrP^C knock mice (Bueler et al., 1992) on a BALB/c background were used (Frigg et al., 2006). Mice with this genetic background were selected because they are homozygous for the retina pigment epithelial protein $Rpe65_{450Leu}$ (Frigg et al., 2006). The RPE65 gene is involved in rhodopsin regeneration in the retina (Redmond et al., 1998) and mice expressing the $Rpe65_{450Leu}$ are more vulnerable to light induced degeneration of the retina (Danciger et al., 2000; Wenzel et al., 2001). Before inducing phototoxicity, the mouse eye was infected with the AAV8 expressing the scFv POM2 via intravitreal injection. To ensure a stable expression of the virus, mice were kept for two weeks before phototoxicity was induced via light exposure. A light dose of 10'000 lux for 2h was used to induce strong photoreceptor apoptosis. 10 days after the induction of the neuronal loss, the mice were sacrificed and the retina collected.

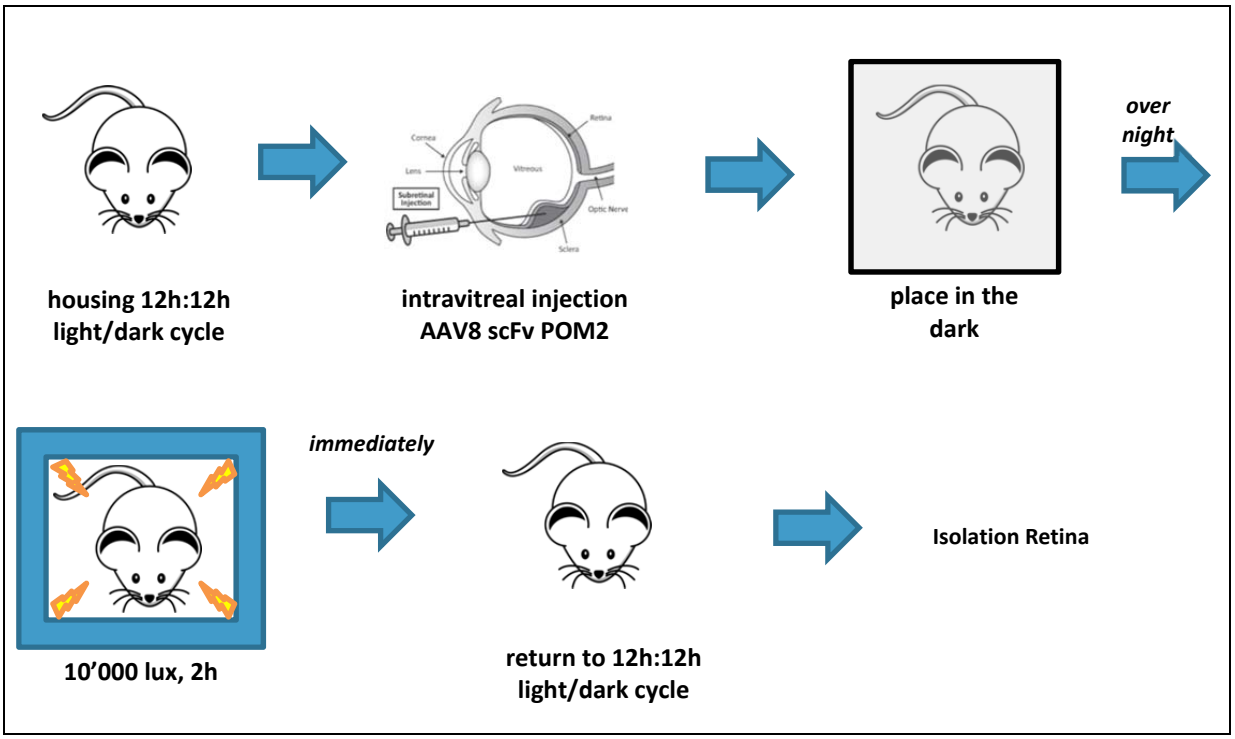


Figure 38: Experimental setup of protective of POM2 against the phototoxicity in the retina
Before the study started, mice were kept in a 12 h:12 h light/dark cyle. The mouse retina was transduced with AAV8 expressing scFv POM2 via intravitreal injection of the virus. To ensure a stable expression of the AAV mice were kept for 2 weeks. Prior to induction of the retinal degeneration with 10'000 lx for 2h mice were placed over night into a dark environment. After light exposure, mice were immediately returned to the 12 h:12 h light/dark cycle and sacrificed after 10 days.
Image intravitreal injection: https://www.sec.gov/Archives/edgar/data/1273636/000119312514094588/g615962g94h06.jpg

The virus should secrete scFv POM2 and express tdTomato in the cytoplasm. The expression of the virus was expected in retinal ganglion and the Müller glial cells (Lebherz et al., 2008; Schon et al., 2015). However, in cryosections of the retina no tdTomato fluorescence was detected. As a His-Tag is attached to the scFv POM2, I stained the retina with an anti-His-Tag antibody to detect the expression of the scFv POM2. From previous studies, it was expected that the AAV would transduce ganglion glial and Müller cells (Lebherz et al., 2008; Schon et al., 2015). None of this cell types were infected and show tdTomato. Independent from the infected cells, a rather unspecific staining was visible in the retinal periphery (see Figure 39). However, as mentioned before, the antibody should be secreted. Therefore, it was expected that most of the antibody is present extracellularly and it is not surprising that this method did not lead to a specific signal.

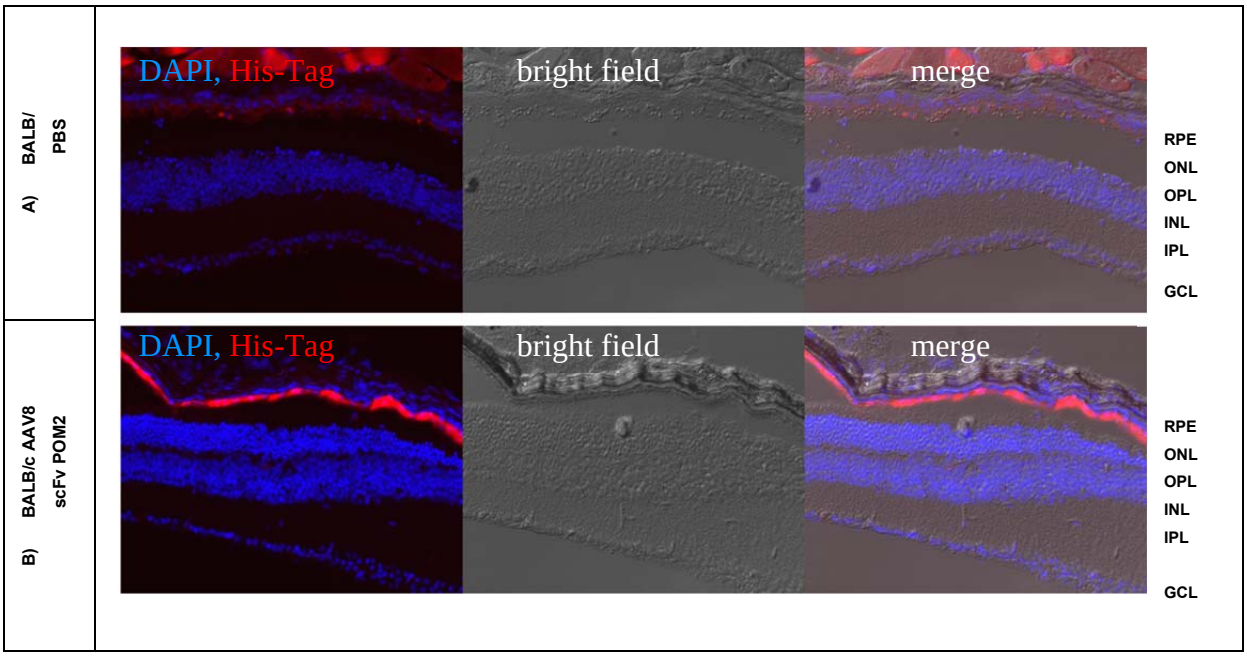


Figure 39: Absence of tdTomato expression in transduced retinas
Cryosections of the mouse eye 24 days after intraventricular injection of A) PBS or B) the AAV8 scFv POM2. It was expected that the AAV will transduce ganglion glial cells in the GCL and Müller cells in the IPL. In both cases a rather unspecific staining of the RPE was obtained, which suggests that the His-Tag staining is not specific.
RPE: retina pigmented epithelium; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer.

Despite the absence of significant viral scFv POM2 expression in the eye, retinal sections were analyzed. The retina morphology of the different samples was compared to evaluate if POM2 influences the light induced photoreceptor apoptosis. The untreated mouse eyes of both genotypes that were injected with PBS only revealed an intact retinal morphology (see figure A, C, E, G). Depletion of the photoreceptors suggested a severe phototoxicity in the Balb/c mice, which could not be rescued via the injection of the AAV8 scFv POM2 (see figure C, D). Interestingly, no PrP^{C} dependent effect could be observed comparing Balb/c and PrP$^{0/0}$ Balb/c mice after light induced photoreceptor apoptosis (see figure G, H). This result contradicts the previous finding that PrP^{C} has a protective effect against light induced photoreceptor apoptosis. Most likely this is caused by the different experimental setup. In this experiment a relatively strong light exposure (10'000 lux, 2h) was used compared to the mild induction of phototoxicity (5'000 lux, 10 min) in the previously published experiment. In addition, the induced photoreceptor mediated apoptosis was so strong that hardly any photoreceptor cells remained. Based on this experimental setup, it could not be evaluated if PrP^{C} is capable of promoting light induced apoptosis and scFv POM2 can be used for neuroprotection.

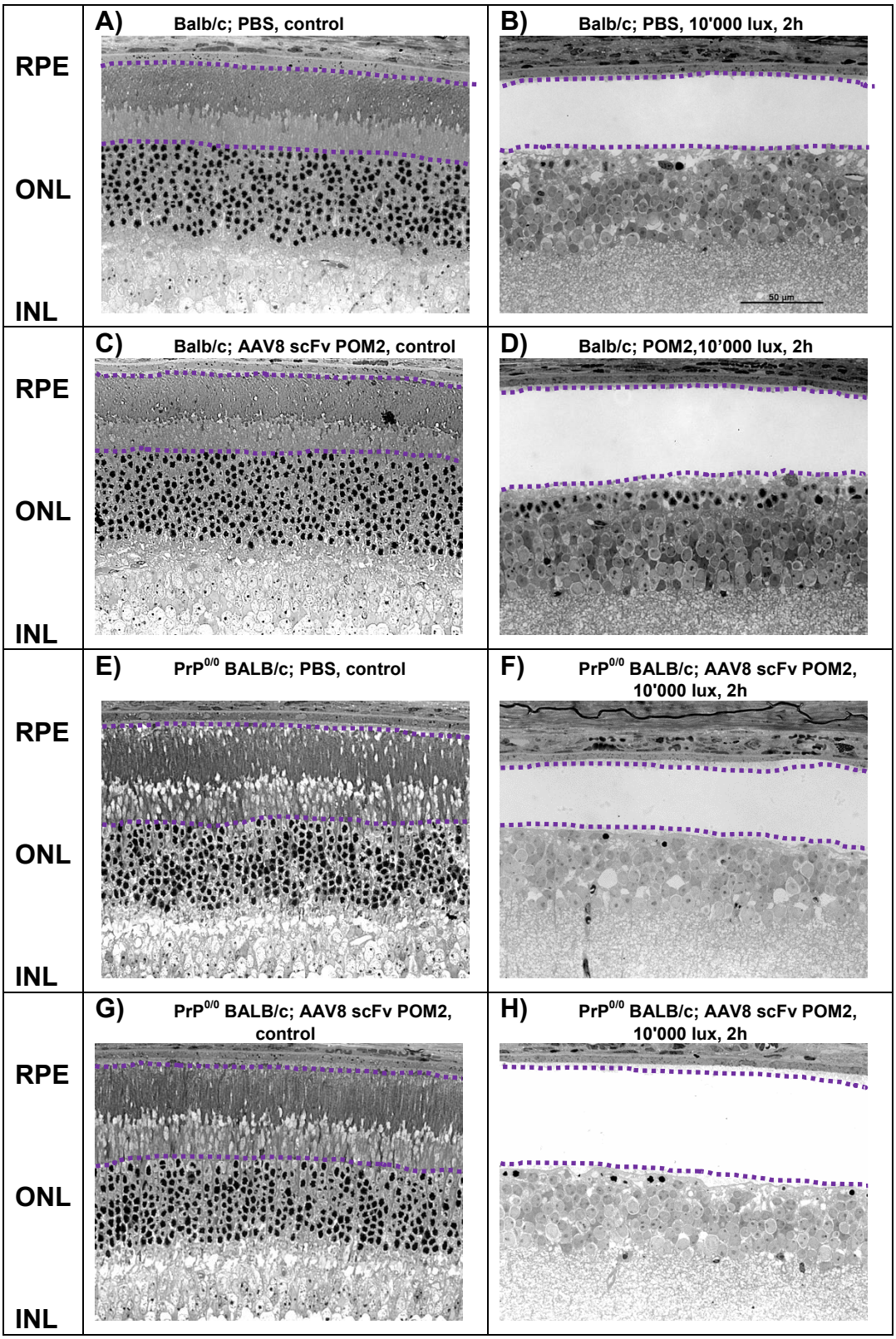


Figure 40: Infection withAAV8 scFv POM2 could not ameliorate light-induced photoreceptor apoptosis

Intravitreal injections with AAV8 scFv POM2 or a mock injection with PBS were done in BALB/c or $PrP^{0/0}$ BALB /c mice. After 14 days phototoxicity was induced and 10 days later, the retina was collected. (A, C, E, G) BALB /c or $PrP^{0/0}$ BALB/c mice show normal retina morphology independent of whether AAV8 scFv POM2 or PBS was injected. (B, D) The light dose of 10'000 lux for 2h caused severe toxicity in BALB/c mice. No protective effect of scFv POM2 could be observed. (G, H) The ablation of PrP^C did not reveal any difference in cell morphology. Purple dotted lines label photoreceptor containing areas.

RPE: retinal pigment epithelium, ONL: outer nuclear layer, INL: inner nuclear layer

6.6. Discussion

Recently, it has been shown that POM2, an antibody binding to the N-terminal region of PrP^C, counteracts neurodegeneration in prion infected and POM1 (a toxic antibody targeting the globular domain of PrP^C) treated brain slices (Sonati et al., 2013; Herrmann et al., 2015). In both model systems, proteins involved in the PERK signaling branch of the UPR are downregulated upon POM2 treatment (Herrmann et al., 2015). ER stress induces the UPR in many PMD's (Hetz and Mollereau, 2014). In addition, it has been discovered that PrP^C is involved in synaptic degeneration in Alzheimer's disease (Lauren et al., 2009). Therefore, it is assumed that PrP^C could act as a general cell death transducer, such that its FT ligand binding antibody such as POM2 could lead to amelioration of disease phenotypes in PMD's.

Distinctive subsets of neurons undergo cell death in different PMDs (Saxena and Caroni, 2011; Jackson, 2014). Therefore, the protective potential of POM2 against ER stress was tested in different types of neurons (see Table 10). Neuronal viability was characterized by the cell number with significant neurite outgrowth. In interneurons no beneficial effect of POM2 against ER stress could be observed while a delay in neurodegeneration was detected in moto- and granule neurons (see Table 10). The effect of POM2 was less pronounced in motoneurons than in granule neurons. The reason for this might be that the motoneurons only express levels of PrP^C comparable wild type. In addition, they were co-cultured with *tga20* astrocytes, which also binds POM2 and thus reduce the amount of the antibody available for binding to neuronal PrP^C. In general, astrocytes have a neuroprotective role (Takuma et al., 2004) and may mask the effect of POM2.

To ensure that the observed effect was PrP^C dependent, the experiment was repeated in primary granule neurons from PrP^C KO mice (see Table 10). In this case, POM2 could not counteract the toxicity induced by ER stress. Therefore, the effect seems to indeed be PrP^C dependent.

Table 10: Protective effect of POM2 against ER stress in different types of neurons

neuron type	PrP^C level	POM2 counteracts changes caused by ER stress		comment
		cell number with significant outgrowth	neurite outgrowth	
interneurons	wild type	no	no	co-culture with tga20 astrocytes
motoneurons	wild type	yes	no	co-culture with tga20 astrocytes
cerebellar granule neurons	overexpression	yes	yes	pure neuronal culture
cerebellar granule neurons	knock out	no	no	pure neuronal culture

Expression levels of specific proteins were analyzed in human neurons pre-incubated with POM2 before ER stressor treatment to explore the underlying mechanism of POM2 to counteract ER stress-induced neurodegeneration. An overview of the results is depicted in Figure 41.

Interestingly, PrP^C levels are significantly upregulated 24h after the induction of ER stress with a high dose of TM. This effect was observed in the breast carcinoma MCF-7 cell line and in human primary cultures (Dery et al., 2013; Misiewicz et al., 2013). This supports our hypothesis that PrP^C plays a crucial role in the UPR (see Figure 41 A). Further studies of this group revealed that the PrP^C promotor contains an ER stress response element (ERSE) that is regulated by XBP1 (Misiewicz et al., 2013). Recently it was shown in skeletal muscle cells that activation of Fyn induces the Ire1 pathway via mTORC1 (Wang et al., 2015). In Alzheimer's disease PrP^C is involved in the activation of Fyn. Assuming that Ire1 signaling in skeletal muscle cells and neurons are similar, it could be hypothesized that UPR induces PrP expression via the Ire1 pathway, whereby PrP activates Fyn, which leads to a further stimulation of Ire1 via mTORC1, thereby creating a positive feedback loop. However, the depletion of XBP1 in prion infected mice does not ameliorate the disease phenotype (Hetz et al., 2008), which indicates that the signaling interactions must be more complex.

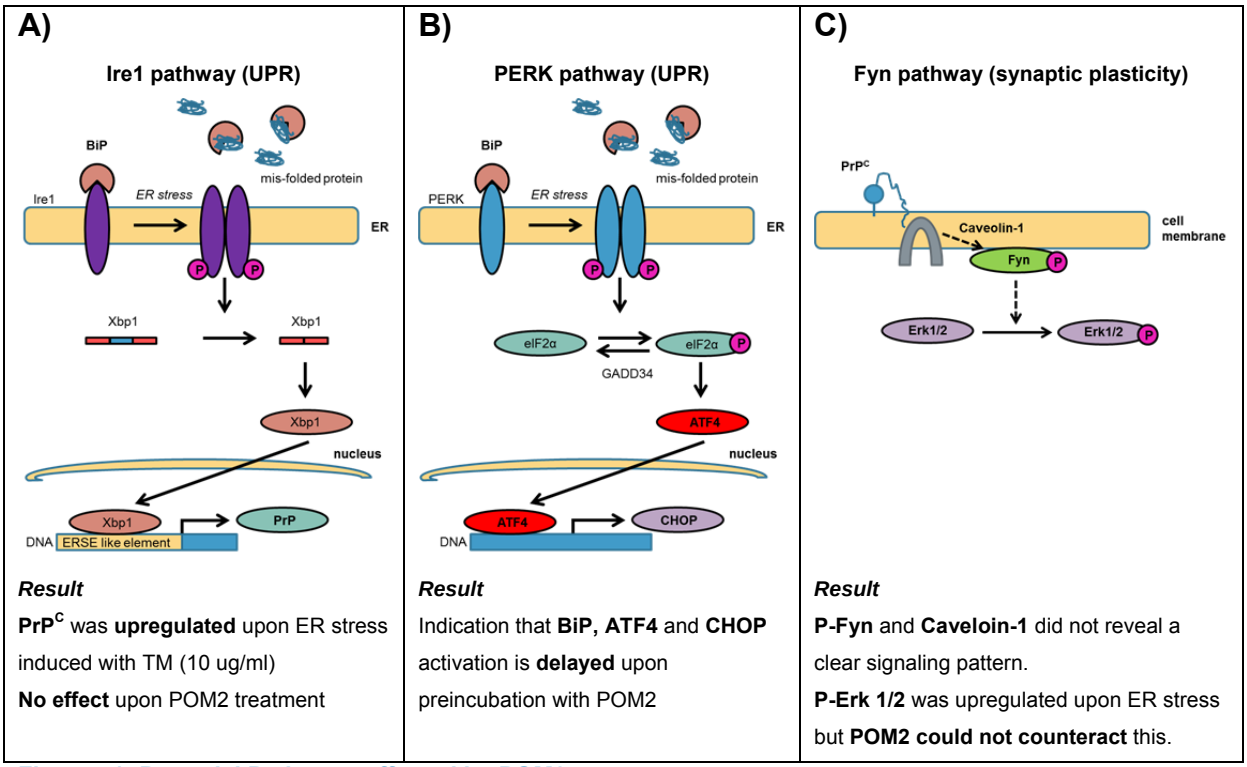
Based on my preliminary data, it seems that POM2 can delay the upregulation of BiP, ATF4 and CHOP upon ER stress induction. This suggests that the PERK signaling branch of the UPR is affected by POM2 treatment (see Figure 41 B). Therapeutic targeting of the PERK pathway has already been applied to prion infected mice. Oral treatment with the PERK inhibitor GSK2606414 was able to counteract

neuropathological and clinical signs (Moreno et al., 2013). However, the disturbed ER homeostasis caused by the drug leads to severe weight loss and mild hyperglycemia (Moreno et al., 2013). Therefore, targeting PERK might be not optimal to counteract the clinical signs of PMD's. Additionally, it has already been shown that, depending on the type of PMD, inhibition of the PERK pathway can either accelerate or attenuate the disease phenotype (Bell et al., 2016; Freeman and Mallucci, 2016).

Further data are necessary to predict the role of PrP in UPR signaling. However, based on the observation that PrP^C knock out mice show no severe phenotype apart from demyelination (Weissmann and Flechsig, 2003; Steele et al., 2007); it is expected that therapeutic targeting of PrP^C to counteract PMD's seems to cause fewer side effects.

Antibody mediated PrP^C activation induces caveolin-1 dependent stimulation of Fyn/Erk1/2 signaling (Mouillet-Richard et al., 2000; Toni et al., 2006; Pantera et al., 2009; Shi et al., 2013). It has been reported that the octapeptide region in particular is involved in the interaction between PrP^C and caveolin-1 (Shi et al., 2013). While ER stress leads to an activation of Erk1/2, no significant changes in the expression levels of caveolin-1, the phosphorylated forms of Fyn and Erk1/2 could be observed. It is known that Erk 1/2 is induced via ER stress but the underlying mechanism behind its activation is not yet resolved (Dery et al., 2013; Darling and Cook, 2014). Based on my data, it seems that the PrP^C/caveolin-1/Fyn interaction is not involved in this pathway.

In summary based on data obtained from the live cell imaging and the translational studies, further studies are needed to elucidate the underlying molecular mechanism.



Result

PrP^C was **upregulated** upon ER stress induced with TM (10 ug/ml)

No effect upon POM2 treatment

Result

Indication that **BiP, ATF4** and **CHOP** activation is **delayed** upon preincubation with POM2

Result

P-Fyn and **Caveloin-1** did not reveal a clear signaling pattern.

P-Erk 1/2 was upregulated upon ER stress but **POM2 could not counteract** this.

Figure 41: Potential Pathways affected by POM2 treatment

A) Upon ER stress BiP is released form Ire1, which induces auto-phosphorylation of Ire1 that in turn activates unconventional splicing of Xbp1 (Hetz, 2012). The transcription factor Xbp1 translocates to the nucleus where it binds to (ERSE)-like elements activing the expression of PrP and other genes (Dery et al., 2013; Misiewicz et al., 2013). B) ER stress releases BiP from PERK (Hetz, 2012). This leads to the auto-phosphorylation of PERK that stimulates the activation of eIF2α, which induces the specific expression of ATF4 that translocates to the nucleus where it induces the expression of CHOP among others(Ron and Walter, 2007). C) PrP^C stimulates caveloin-1, which then leads to the phosphorylation of Fyn that via the MAPK pathway activates Erk1/2 (Mouillet-Richard et al., 2000; Toni et al., 2006; Pantera et al., 2009; Shi et al., 2013).

According to previous data from our lab (Prof. Magdalini Polymenidou, unpublished), intravenous injection of POM2 causes hemolysis in a PrP^C dependent manner. This effect is most likely caused by antibody mediated complement activation (Walport, 2001; Dunkelberger and Song, 2010) that leads to lysis of the red blood cells. However, to confirm this finding, further studies are necessary, that for example assesses levels of complement component 3 (C3), a protein that is specifically upregulated after complement activation (Walport, 2001; Carroll, 2004).

The observation that intravenous injection of POM2 induces hemolysis, excludes intravenous administration as a viable method for antibody delivery. Therefore, an AAV8 expressing scFv POM2 that lacks the Fc region responsible for complement activation (Noris and Remuzzi, 2013; Melis et al., 2015) was generated. Compared to injection of the therapeutic antibody, the viral delivery has several advantages. Only one administration is necessary to allow continuous expression of the virus secreting scFv POM2, while repeated injection of the antibody or Alzet pump would be

required, since it is rapidly degraded (Worn and Pluckthun, 2001). This will reduce the stress caused in mouse models. In addition, huge amounts of antibody would be necessary for intravenous application because only a small proportion of the antibody can cross the blood brain barrier (Martins et al., 2016). Therefore, such studies would be quite expensive. Another advantage of the virus is the continuous expression of the therapeutic agent, in contrast to intravenous application, which causes fluctuations in the POM2 blood plasma level and may thereby reduce the efficiency.

Expression of the virus was tested in brain slices in wild type mice. These studies showed that the fluorescent marker labels the infected cells and that scFv POM2 secretion occurs in amounts sufficient to counteract RML and POM1 mediated neurodegeneration in slices.

To test the protective potential in vivo, AAV8 scFv POM2 was applied to an *in vivo* model of light induced photoreceptor apoptosis in collaboration with Prof. Christian Grimm. Transduction of the retina with the AAV8 scFV POM2 did not show any protective effect against light induced phototoxicity in the retina. There are several explanations for the absence of a therapeutic effect. Based on the design of the virus and our data from the slices, the transduced cells should express the fluorescent marker tdTomato. In fact, cryosections of the retina do not reveal any red labeling. Detection of the scFv POM2 antibody was attempted with an anti-His antibody, because a His-Tag is attached to scFv POM2; however, this resulted in only an unspecific staining, which is not surprising because scFv POM2 is readily secreted and only a small proportion can be found within cells. It is possible that the virus did not transduce the retina efficiently or the signal was depleted during the processing of the cryosections. In any case, the retinas were further processed for morphological analysis. A significant loss of photoreceptors after light exposure in the Balb/c as well as Balb/c $PrP^{0/0}$ mice was observed. However, POM2 was not able to counteract the light induced toxicity. In addition, depletion of PrP^C did not lead to significant changes in photoreceptor loss.

The light induced photoreceptor damage is caspase-1 dependent (Grimm et al., 2000). Recent results from our lab suggesting that neuronal loss in prion and POM1 mediated toxicity is rather Calpain dependent (Sonati et al., 2013; Herrmann et al., 2015). Based on the different pathways responsible for neurodegeneration, participation of PrP^C in cell death signaling might change. This would also explain

that mild photoreceptor mediated apoptosis could be prevented by overexpression of PrP^C (Frigg et al., 2006).

In general, further studies and data are necessary to confirm our hypothesis that PrP^C could act as a cell death transducer in PMD's.

6.7. Outlook

My preliminary data indicate that POM2 may delay ER stress mediated neurodegeneration. To elucidate this hypothesis, primary or ESC derived neurons could be transduced with a lentivirus expressing a reporter gene like luciferase or GFP under the control of regulatory elements that are affected during ER stress. A possible target is the ERSE, which is mainly involved in the ATF6 and Ire1 UPR signaling branches (Samali et al., 2010). To explore the involvement of the PERK signaling branch in more detail, an ATF4 reporter lentivirus that expresses the reporter gene under the regulatory elements of ATF4 (ATF4 5′UTR containing the uORF1 and uORF2 fused to the ATF4 initiation codon) could be used (Inageda, 2010).

The assessment of the therapeutic potential of POM2 in an animal model of photoreceptor mediated apoptosis did not reveal a clear result. Therefore, in a next step, gene therapy with the AAV8 scFv POM2 should be performed directly in mouse models of PMD's such as prion disease, Alzheimer's disease, ALS and Huntington's disease.

Before POM2 gene therapy is applied to animal models, the protective effect of POM2 should be tested in *in vitro* systems of the aforementioned PMD's. Prion organotypic slice culture assay represent a feasible model system (Falsig et al., 2008; Falsig et al., 2012), for ALS, SOD1 G93A mouse embryonic stem cells (mESCs) can effectively model the disease phenotype (Di Giorgio et al., 2007). In the case of Alzheimer's disease, possible models include primary neuronal cultures or cell lines treated with neurotoxic amyloid-beta oligomeric peptides (Klein, 2002; Benilova et al., 2012) and in Huntington's disease, one of the established *in vitro* cell model systems (Cisbani and Cicchetti, 2012).

After the successful application of POM2 to *in vitro* models of PMD's, AAV should be applied post-symptomatically in the respective mouse model. The potential of scFv POM2 to ameliorate the disease phenotype should be characterized according to the disease model. For this aim, behavioral studies like Rotarod, foot print analysis, novel

object recognition test, survival time and pathophysiological studies could be used (see Figure 42). For mouse models where POM2 has a beneficial effect, post-symptomatic studies should be performed as well.

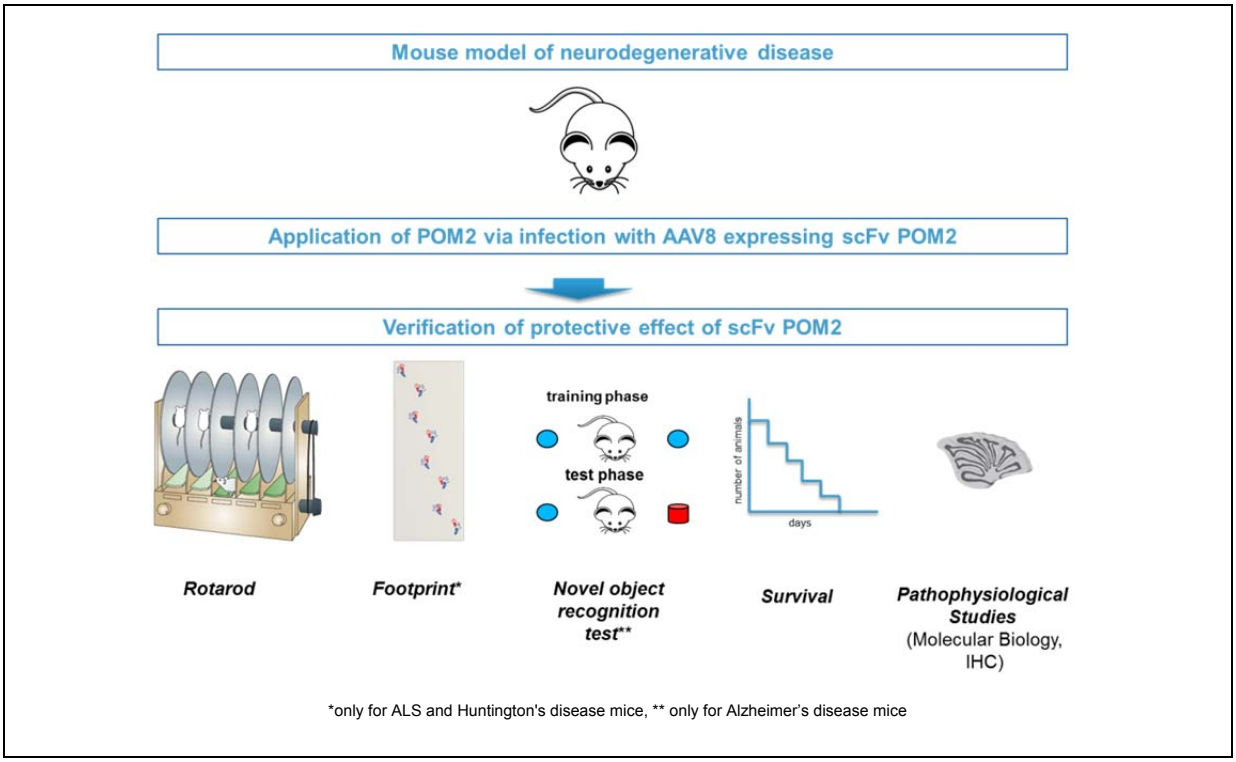


Figure 42: Therapeutic treatment of neurodegenerative mouse models with scFv POM2
Mouse models of several neurodegenerative diseases like prion disease, ALS, Alzheimer's and Huntington's should undergo gene therapy with scFv POM2. To evaluate the potential of POM2 to ameliorate the disease phenotype, behavioral studies like Rotarod, footprint analysis, novel object recognition test or survival and pathophysiological studies should be assessed.
(image Rotarod and Footprint: (Brooks and Dunnett, 2009))

Currently there is no cure for the selected PMD's. Involvement of PrP^C in the pathology of these diseases would represent a pioneering discovery in this field and reveal the potential of PrP^C as a new therapeutic target for neurodegenerative diseases.

6.8. Material and Methods

All reagents are purchased at Thermo Fischer if nothing else is stated.

6.8.1. Mice

The housing of mice and animal experiments were performed according to Swiss Animal Protection Law and in compliance with the regulations of the veterinary office (canton Zurich). For our studies the following mice were included *Prnp*$^{ZH1/ZH1}$ mice

(Bueler et al., 1992), *Prnp*$^{ZH3/ZH3}$ mice (Nuvolone et al., 2016), C57BL/6-*tga*20 mice (McHugh et al., 2012), B57BL/6J (The Jackson laboratory), Balb/c (Harlan laboratories), $Prnp^{0/0}$ Balb/c (Frigg et al., 2006).

6.8.2. Primary cell culture

6.8.2.1. Cerebellar granule neurons

To generate cerebellar granule neurons, a modified protocol from the Manzini group was used (Lee et al., 2009). In detail, 4 to 6 days old pups were decapitated and put in dissection buffer (6.7ml Glutamax 45%, 5ml P/S, 50 ml HBSS ad up to 500 ml with sterile water). The head was fixed with forceps and the brain was accessed by inserting micro dissecting scissors into the foramen magnum and cutting straight toward the eyes. With the help of forceps the skin was removed and the skull was lifted up to expose the brain. After pinching off the cerebellum and surrounding midbrain, the tissue was transferred into a new plate with dissection buffer. The cerebellum was separated from the midbrain, brain stem and choroid plexus. After gentle removal, the meninges were peeled off and the cerebellum was transferred into a plate with fresh dissection buffer. For digestion of the tissue the dissection buffer was removed and 1 ml papain (Worthington) and 125 ul DNase (Worthington) (for 1-3 cerebelli) were added and incubated for 15 min at 37°C. Next the cells were triturated and passed to a 70 um cell strainer to remove cellular debris. To separate the viable from death cells, the cell suspension was loaded onto 5ml Ovomucoid inhibitor (Worthington) and a gradient centrifugation at 500 rcf for 5 min was performed. After removal of the supernatant the remaining viable cells were dissolved in cerebellar granule neuron media (Neurobasal media containing 1x GlutaMAX, 1x P/S, 1x B27 supplement and 250 uM KCl) and plated on Lamin/Poly-D-Lysine coated plates.

6.8.2.2. Primary astrocytes culture

5.8.2.3

6.8.3. Plating embryonic stem cells (ESCs)

Irradiated mouse embryonic fibroblasts (MEF) feeder cells (amsbio, GSC-6001G) were seeded on plates pre-coated with gelatin (Millipore, #ES-006-B) in MEF-media (DMEM, 50 % fetal bovine serum (FBS), 1% penicillin/streptomycin, 1 % Glutamax,

1 % Non-essential amino acids (NEAA)). mESCs were cultured on this plates in mESC-media (Knockout D-MEM, 90 ml ES Cell FBS, 6 ml beta-mercaptoethanol, 6 ml NEAA, 1 % penicillin/streptomycin, 1 % Glutmax, 1'000-2'000 units/ml leukemia inhibitory factor (Lif)).

6.8.4. Preparation of astrocytes for sorting mESCs

See 5.8.5

6.8.5. Derivation of neurons from mESCs

See 5.8.6

6.8.6. FACS-sorting

5.8.7

6.8.7. Derivation of neurons from iPSC derived neurons

5.8.8

6.8.8. Estimation of serum hemoglobin using o-toluidine

For estimation of the hemoglobin concentration, the Goyal and Basak protocol was used (Goyal and Basak, 2009). In brief 100 ul of o-Toluidine solution (0.4 g o-toluidine dissolved in 20 ml glacial acetic acid and 80 ml Ethanol) were mixed with 100 ul hydrogen peroxide solution (6.67 ml hydrogen peroxide (30 % (v/v)) add up to 100 ml with analytical water and add 2.26 gm sodium acetate). After an incubation time of 5 min 1 ul of the hemoglobin solution were added, mixed and followed by a subsequent kinetic measurement at 630 nm for 180 sec. The rate of the kinetic reaction is directly proportional to the hemoglobin concentration (Goyal and Basak, 2009)

6.8.9. Organotypic slices

Organotypic cerebellar slices were prepared according to a modified protocol from Falsig et al. (Falsig et al., 2008; Falsig et al., 2012). In brief, 300 µm thick sections of cerebella from 13 day old pups were cut with the vibratome and kept in Gey's balanced salt solution (GBSS) (NaCl 8 g l–1, KCl 0.37 g l–1, Na_2HPO_4 0.12 g l–1, $CaCl_2$ $2H_2O$ 0.22 g l–1, KH_2PO_4 0.09 g l–1, $MgSO_4$ $7H_2O$ 0.07g l–1, $MgCl_2$ $6H_2O$

0.210 g l–1, $NaHCO_3$ 0.227 g l–1) supplemented with the glutamate receptor antagonist kynurenic acid (1 mM) at 4°C. For roller tube cultures the slices were embedded in a plasma clot on a coverslip pre-coated with Poly-D-Lysine (ref). 2 slices were plated per coverslip.

6.8.10. Transduction organotypic slices

For viral infection, brain slices were incubated in 500 ul slice-culture medium" (50% vol/vol MEM, 25% vol/vol basal medium Eagle and 25% vol/vol horse serum supplemented with 0.65% glucose (w/vol), penicillin/streptomycin and glutamax (Invitrogen)) containing a defined amount of AAV (titer 10^8-10^{11}). The slices were kept at 1h at 4°C before they were put into a specific incubator that contains a rotating wheel for the tubes. After 2 days 250 ul additional slice-culture media were and complete media was exchange every week.

6.8.11. Morphological analysis

5.8.11

6.8.12. Western Blot

See 5.8.12

Antibodies used: ATF4 (CST, # 11815), BiP (CST, #3183), Caveloin-1 (CST, #3238), CHOP (CST, #5554), P-Erk1/2 (CST, #4370), P-Fyn (abcam, # ab182661), POM1 (Polymenidou et al., 2008),

The following blocking buffers were used:

- 5 % w/v Albumin IgG free, 1 x TBS (20 mM Tris-Base, 150 mM NaCl), pH =7,4, 0.1 % Tween-20 (ATF4, BiP, CHOP, P-Erk1/2),
- 5 % w/v milk, 1 x PBS, 0.1 % Tween (POM1, Caveloin-1)
- 5 % w/v TopBlock, 1 x PBS, 0.1 % Tween (P-Fyn)

6.8.13. ELISA for determination of scFv POM2 in the media supernatant

A 96 well tissue culture plate was coated with 50 ul/well of rPrP (3 ug/ml) in 50 mM Tris. After incubation overnight at 4°C, the plate was washed 5 times with washing buffer (PBS, 01.% Tween) and 100 ul/well of blocking buffer (5% w/v TopBlock, 0.1% Tween-20) was added and incubated for 2h at room temperature. After removal of

the blocking buffer the samples were added and incubated for 2h at room temperature. The plate was washed 6 times with washing buffer before 50 ul/well Anti-6X His tag® antibody (Biotin) (ab27025) (1:1'500) in sample buffer (= slice culture media) was added. This step was followed by 6 times washing with washing buffer 50 ul/well of streptavidin-HRP (#554066, Becton Dickinson AG) 1:1000 in sample buffer was added and incubated for 1h at room temperature. After 6 times washing with washing buffer 50 ul/well chromogen (BIOSOURCE 16A8/1) was added and incubated up to 30 min. 50 ul/well stop solution (H_2SO_4 0.5 M) was added and plate were measured at 450 nm.

6.8.14. Phototoxicity

Viral infection of the mouse eye and the light induced phototoxicity experiment was performed by the group of Prof. Christian Grimm. Before the study started the mice were kept in a 12 h:12 h light/dark cycle. The mouse eye was infected with the AAV8 expressing scFv POM2 via intravitreal injection of the virus. 1 µl AAV8 expressing scFv POM2 was injected in the left eye and 1 µl PBS was injected in the right eye of one mouse. The mice were then kept for 2 weeks to ensure stable expression of the virus. After dark adaptation overnight, the pupils were dilated and the mice were exposed to 10'000 lux for 2h. After light exposure, the mice were immediately returned to the 12 h:12 h light/dark cycle and sacrificed after 10 days.

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- System Life Cycle (SLC) documentation
- Evaluation of SLC documentation (air-technical systems, clean rooms, building management system)
- Optimization of the Standard Operating Procedures (SOPs)

03/2008 – 03/2010 **Project engineer at Steinbeis Pharmatechnik GmbH, Irndorf, Germany**
Operated for ***Boehringer-Ingelheim Pharma GmbH & Co. KG,*** Biberach, Germany

- Preparation of qualification management documentation according to the EU-GMP Guide for air-technical systems
- Training of external persons in SOPs
- Organization and coordination of meetings
- Evaluation of SLC documentation (biotechnical pharma production)

09/2006 – 02/2007 **Diploma thesis, Roche Diagnostics GmbH, Penzberg, Germany**
Subject: Derivatization and valuation of surfaces for the immobilization of enzymes

School education and job training

09/2001 – 08/2003 **Fachoberschule (field: engineering), Memmingen, Germany**
Graduation: University of applied sciences entrance qualification

09/1998 – 07/2001 **Management assistant in office communication, City administration Memmingen, Germany**
Graduation: Assistant in office communication

Poster presentations

08/2015 *Retreat of the MLS PhD Program, Engelberg (CH)*
Poster presentation: Identification and characterization of PrP^C as a cell death transducer

06/2015 *Annual meeting of the Swiss Stem Cell Network (SSCN), Basel (CH)*
Poster Presentation: PrP^C as a general cell death transducer

06/2014 *Day of Clinical Research University Hospital Zurich, Zurich (CH)*
Poster presentation: Characterizing non-cell autonomous neurotoxicity in prion disease

09/2013 *Retreat of the MLS PhD Program, Nidwalden (CH)*
Poster presentation: Role of Glia in Prion Neurotoxicity

Publication

Cappello, S., Gray, M.J., Badouel, C., Lange, S., ***Einsiedler, M.,*** Srour, M., Chitayat, D., Hamdan, F.F., Jenkins, Z.A., Morgan, T.*, et al.* (2013). Mutations in genes encoding the cadherin receptor-ligand pair DCHS1 and FAT4 disrupt cerebral cortical development. Nature genetics *45*, 1300-1308.